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It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

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HIGHLAND PARK, ILL.

A STUDY OF THE DECOMPOSITION PRODUCTS OF SPORE-BEARING BACTERIA IN HEATED MILK[1]

HENRIETTA LISK

Received for publication June 22, 1923

INTRODUCTION

Years ago it was pointed out by Flügge (1894) that milk contains resistant spores of many species of bacteria which are able to bring about putrefactive changes under suitable conditions. Some of these spores were extremely resistant to the action of heat, withstanding boiling for long periods, eight hours or more. Flügge suggested that such spores taken into the alimentary canal of children would withstand the destructive action of the gastric juice, pass through the small intestine, multiply in the large intestine and there produce putrefactive products which might act as an irritant to the intestinal mucosa and thus play a rôle in the production of summer diarrhea. It was subsequently shown by Ford and Pryor (1904) that in decomposing heated milk the aerobic spore-bearing bacteria described by Flügge were always present, and these species were carefully described by Lawrence and Ford (1916). It was also shown by Ford and Williams (1919) that heated milk kept in a warm place (37°) decomposes with the elaboration of products poisonous to animals, and some of these products were traced to the growth of *Bacillus Welchii* (*Bacillus aerogenes capsulatus*). In a subsequent discussion of the products of putrefaction from several anaerobes it was pointed out by Wolf and Harris (1916–1917) that no adequate study of the products of putrefaction from aerobic spore-bearing bacteria had ever been made.

[1] Thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Science in Hygiene in the School of Hygiene and Public Health, the Johns Hopkins University, 1923.

In view of the universal use of cow's milk as a food for man, especially for children, and in further consideration of the constant presence of such organisms in milk as shown by Flügge and Ford and Pryor, this investigation was undertaken to determine the nature and extent of the decomposition of milk caused by the predominant aerobic spore-bearing bacteria found in cow's milk in Baltimore.

EXPERIMENTAL WORK

Source of material

As a preliminary to the quantitative determination of the decomposition products formed in milk by aerobic spore-bearing bacilli, a study of the flora of pasteurized milk was carried out. Samples of pasteurized milk, varying in amount from 300 to 400 cc. were placed in water baths and subjected to a temperature of 70°C. and 80°C. for thirty minutes. At the end of this time the flasks of milk were placed in the incubator at 37°C. and allowed to remain for four or five days. The milk was then plated and the plates were incubated for forty-eight hours or more, at 37°C.

The aerobic spore-bearing bacteria which were isolated were identified according to recent investigations. The following spore-bearing bacilli were isolated from Baltimore milk: *Bacillus cereus*, *Bacillus albolactus*, *Bacillus mesentericus-vulgatus*, *Bacillus mesentericus-fuscus*, *Bacillus subtilis-viscosus*, *Bacillus simplex* and *Bacillus circulans*. From a sample of raw milk from Fort McCoy, Florida, subjected to a temperature of 80°C. for thirty minutes, the following additional organisms were isolated: *Bacillus megatherium* and a spore-bearing bacillus resembling *Bacillus brevis*, as described by Ford, in its morphology and in some of its cultural reactions. Contrary to the production of alkalinity in glucose, lactose and sucrose broth, as described in cultures of *Bacillus brevis*, this organism forms slight amounts of acid in sugar broths. Milk is softly coagulated on the eighth day. Milk cultures of this bacillus have an unpleasant odor, resembling that of codfish. A gas-producing aerobic spore-bearing bacillus was also isolated from the samples of Florida milk and was identified as *Bacillus asterosporous* of Myer.

Of the aerobic spore-bearing bacilli thus isolated, only those which digest milk were used in the later chemical work. The organisms selected for chemical investigation were *Bacillus cereus*, *Bacillus albolactus*, *Bacillus mesentericus-vulgatus*, *Bacillus mesentericus-fuscus*, *Bacillus megatherium*, *Bacillus simplex*, *Bacillus subtilis-viscosus*, and *Bacillus brevis*. The same strain of each bacillus was used throughout the series of analyses of the decomposition products formed in milk by the action of this bacillus.

One cubic centimeter of a plain milk culture of the bacillus under study was introduced into a liter flask containing 400 cc. of sterile milk. The flask was then incubated at 37°C. for eight days or for twenty days, and, at the end of the incubation period, the following changes produced by the action of the bacteria were determined: (1) Change in ammonia content, (2) change in amino nitrogen content, (3) change in lactose content, (4) change in hydrogen-ion concentration, and (5) change in volatile-acid content.

Methods used

In determining the ammonia content of sterile milk and milk cultures of the aerobic spore-bearing bacilli under study, the vacuum distillation method, as described by Shaffer (1903), was used.

In the determination of amino nitrogen in sterile milk and milk cultures, the Van Slyke method was used. Two methods of preparing the amino solution were used as a means of placing a check on the technique of the operation of the Van Slyke apparatus. Following the method described by Harris (1919–1920), the residue left in the distilling flask after the distillation of ammonia was acidified with acetic acid and filtered. The filtrate was then made up to 50 cc. and 1 cc. of this amino solution was used for the determination of amino nitrogen in the Van Slyke micro-apparatus. A second method used was that devised by Denis and Minot (1919) for the determination of amino nitrogen in milk. Duplicate solutions were prepared according to both methods and duplicate determinations were made from each solution.

In determining the lactose content of sterile milk and milk cultures of aerobic spore-bearing bacilli, the method used was that devised by Folin and Denis (1918) for the determination of lactose in milk. Denis states that this quantitative sugar method, as well as any other titration method, will give reduction not only with lactose but with any other reducing bodies present, and that it is possible that the action of bacteria on either protein or lactose might give rise to reducing bodies such as aldehydes, ketones and acids. In recording the results obtained by the titrations of milk cultures of bacteria, therefore, they have been designated as reducing substances, calculated as per cent of lactose.

The hydrogen-ion concentration of each sample of sterile milk and of each bacterial culture was determined colorimetrically by comparison with sets of standard solutions containing phenolsulphone-phthalein and brom cresol purple as indicators.

Total acidity was determined by titration of a 1:10 dilution of the medium with N/10 potassium hydroxide, using phenolphthalein as an indicator.

The presence of volatile acids in sterile milk and milk cultures was determined by the steam distillation method of Dyer (1916), as modified from that of Ducleaux (1900). One hundred cubic centimeters of milk or culture were made acid to Congo red paper and steam distilled. The distillation was carried out at a uniform rate for each experiment and the same amount of distillate collected (500 cc.). Harris (1919–1920) states that, except for acids of low volatility, such as formic acid, this method yields from 90 to 95 per cent of the total acid present. The distillate was then titrated with N/10 potassium hydroxide, using phenolphthalein as an indicator. As the amounts of volatile acids in milk, and the amounts formed by the aerobic spore-bearing bacilli, were very small, 1 cc. pipettes were used for the titrations.

In the identification of the volatile acids in milk and milk cultures of the bacteria studied, Dyer's qualitative color tests were used.

Tests for indol were made with Ehrlich's reagent.

Hydrogen sulphide and mercaptan formation were tested for by the method given by Hawk (1917). In the culture flask was

inserted a cork furnished with a glass tube to which was attached a wash bottle containing a 3 per cent solution of mercuric cyanide. The culture was then incubated for thirty days at 37°C.

RESULTS

Bacillus cereus

In table 1 are shown the changes produced in the ammonia, amino acid, lactose, and the total and volatile acid content of milk by *Bacillus cereus* in eight days and in twenty days. Each of the seven analyses was made at a different times, but conditions differed only in the age of the strain and in the age of the seed culture.

A constant, progressive increase in ammonia and amino nitrogen formation was observed. Reducing substances calculated as lactose increased in eight days, but in twenty days a decrease was shown. As stated in the discussion of methods, any reducing bodies formed by the action of the bacteria would be recorded in the determination of lactose. The total acidity, as shown by titration, increased in eight days and in twenty days. The hydrogen-ion concentration, on the contrary, showed a decrease in eight days and in twenty days. Clark and Lubs (1917) state that an *increase* in "reaction," as determined by titration, is not at all incompatible with a *decrease* in hydrogen-ion concentration, the increase in titratable acidity of the milk being due to the hydrolysis of the proteins and the consequent increase in the buffer action against added alkali.

No increase in any individual volatile acid could be demonstrated in the distillate from the culture, with the exception of acetic acid. Tests for butyric and caproic acids were positive, but the intensity of the reactions was no greater than in most samples of sterile milk.

Indol tests were positive in twenty-day cultures both in the acid distillate obtained from the distillation of volatile acids and in the neutral distillate obtained from the cultures by the steam distillation of the medium unacidified. Indol formation was therefore indicated, for the neutral distillate of sterile milk did

TABLE 1

	BACILLUS CEREUS							BACILLUS ALBOLACTUS			BACILLUS MEGATHERIUM							BACILLUS SIMPLEX			
	8 days of growth					20 days of growth		8 days of growth		20 days of growth	8 days of growth					20 days of growth		8 days of growth			20 days of growth
	A	B	C	D	E	F	G	A	B	C	A	B	C	D	E	F	G	A	B	C	D
† Nitrogen as ammonia	3.92+	4.2+	3.92+	6.72+	4.2+	12.32+	12.30+	3.36+	2.80+	1.4+	1.12+	2.80+	1.96+	1.12+	5.48+	4.48+	5.52+	0.84+	1.12+	1.4+	7.28+
† Amino nitrogen (Denis and Minot)	38.0+	23.67+		25.74+	29.08+	57.78+	60.83+	25.71+	21.18+	31.80+		26.51+	24.20+	19.48+	41.33+	44.38+	70.20+	0.67+	2.09+	3.07+	23.48+
† Amino nitrogen (Harris)		24.76+	36.69+	30.36+			85.27+			33.55+				21.86+		46.92+	73.64+	0.62+		3.05+	54.95+
‡ Reducing substances		0.11+	0.27+	0.72+	0.11+	0.11−	0.7−	0.9−	0.47+	2.07−	0	0	0	0	0.21+	0.8+	1.0+	0.07−	0.5−	0.17+	0.04−
pH				0.3−		1.2−	1.0−	1.0+	1.1+	1.1+		0.2−	0.2−			0.1−	0.1−	0.2−	0.2−	0.3−	1.2−
§ Total acid	3.2+	2.4+	2.0+	2.2+	3.4+	2.0+	7.0+	5.6+	3.6+	5.0+	0.6+	0.8+	2.0+	2.0+	2.6+	4.0+	5.0+	0.2−	0.6−	0.2−	1.6+
§ Volatile acid	1.0+	1.4+	1.3+	0.2+	1.0+		3.4+	1.0+	1.4+	2.9+	0	0	0	0	1.5+	2.4+	2.0+	0	0.6−	0	0.8+
Age of seed culture in days			48	11	19		2		26			48	3	8			15	8	17	7	15
Number of samples of milk	V	VI	XI	XIX	XX	II	XXIV	IX	XIX	X	VII	XII	XV	XVI	III	IV	XIX	XIV	XVII	XIX	XXV

The sign + indicates an increase in the content of the medium.
The sign − indicates a decrease in the content of the medium.
† Milligrams per 100 cc. of the medium.
‡ Calculated as percentage of lactose.
§ Cubic centimeters of n/10 KOH required to neutralize 100 cc. of the medium.

not give the indol test. No hydrogen sulphide or mercaptan were formed in twenty days.

Bacillus albolactus

In table 1 the changes produced in milk by the action of *Bacillus albolactus* are also shown. The three analyses were made at different times.

Nitrogen as ammonia formation was constant, but not progressively so. Amino nitrogen showed a progressive increase.

Reducing substances calculated as lactose fluctuated from a decrease to an increase in eight days, and in twenty days showed a decrease. Hydrogen-ion concentration and total acidity, as shown by titration, increased. Volatile acids were formed by *Bacillus albolactus*, and the presence of acetic and butyric acid was recognized in the distillate of the culture. The intensity of the color reactions, however, did not distinguish the volatile acid content of the culture from that of the original medium. No indol, hydrogen sulphide, or mercaptan formation through the action of *Bacillus albolactus* could be demonstrated.

Bacillus megatherium

The decomposition products formed by *Bacillus megatherium* are shown in the third column of table 1. The seven analyses were made at different times.

The formation of nitrogen as ammonia, and of amino nitrogen, was constant and increased progressively. There was no increase in reducing substances in eight days, but a constant increase in twenty days. Hydrogen-ion concentration showed a decrease in eight days and in twenty days, while titratable acidity increased. There was no increase of volatile acids in eight days, but in twenty days there was an increase. The presence of acetic and butyric acid was recognized in the distillate of the culture, but the intensity of the color reactions was not perceptibly greater than in sterile milk. No indol, hydrogen sulphide or mercaptan formation was observed.

Bacillus simplex

The changes in the composition of milk produced by the action of *Bacillus simplex* are shown in the last column of table 1. The four analyses were made at different times.

Increase in nitrogen as ammonia and amino nitrogen was progressive. As in the case of *Bacillus cereus*, *Bacillus mesentericus-vulgatus*, and *Bacillus mesentericus-fuscus*, the amount of amino nitrogen formed in twenty days was much larger when determined by the Harris method than when determined according to the method of Denis and Minot. Some factor evidently operated in the Harris method which did not enter into the Denis and Minot method of determining amino nitrogen. There was possibly a hydrolysis of some of the protein of the culture during the removal of ammonia, thus causing the apparent formation of a larger amount of amino nitrogen when determined by Harris' method.

Reducing substances calculated as lactose were decreased in two eight-day cultures but increased in a third. In twenty days a decrease was noted. Hydrogen-ion concentration decreased in eight days and in twenty days. Titratable acidity also decreased in eight days, but in twenty days increased. Acetic acid was present in the distillate of the culture but not in sufficient amounts to distinguish the culture from the original medium. No indol, hydrogen sulphide or mercaptan formation could be demonstrated by the action of *Bacillus simplex*.

Bacillus mesentericus vulgatus

In table 2 the changes produced in milk by *Bacillus mesentericus-vulgatus* are given. All of the analyses were made at different times.

Ammonia formation and amino nitrogen formation were progressively constant. There was an increase of reducing substances in eight days and in twenty days. Hydrogen-ion concentration decreased in all cultures, while "reaction" increased throughout. Volatile acid formation increased both in eight days and in twenty days. Traces of acetic acid were found in the distillate of the culture.

TABLE 2

	BACILLUS MESENTERICUS-FUSCUS					BACILLUS MESENTERICUS-VULGATUS					BACILLUS SUBTILIS VISCOSUS				BACILLUS BREVIS			
	8 days of growth			20 days of growth		8 days of growth			20 days of growth		8 days of growth		20 days of growth		8 days of growth		20 days of growth	
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	A	B	C	D
† Nitrogen as ammonia	1.40+	4.08+	1.68+	8.40+	8.40+	0.56+	1.12+	1.68+	9.24+	10.08+	0	3.36+	9.52+	10.64+	5.32+	5.6+	7.84+	8.68+
† Amino nitrogen (Denis and Minot)		19.34+	14.21+	26.23+	20.76+	22.57+	44.40+	41.38+	97.36+	93.76+	54.27+	57.23+	92.03+	97.98+	25.54+	25.95+	34.52+	35.49+
† Amino nitrogen (Harris)	20.92+		14.60+	81.77+	74.75+	22.17+	44.8+	46.38+	128.57+	126.87+	56.22+	57.81+	105.91+	109.13+	28.27+	30.49+	32.31+	31.60+
‡ Reducing substances	0.35−	0.27+	0.38−	0	0	0.06+	0.23+	1.16+	1.35+	1.37+	0.99−	0.36−	0.54−	0.92−	0.66−	0.66−	1.0+	1.22+
pH	0.2−	0.1−	0.2−	0.2+	0.2+	0.4−	0.2−	0.5−	1.1−	1.1−	0.4+	0.4−	0.7−	0.6−	0.7−	0.6−	0	0
§ Total acid	0.6+	0.5+	0.6+	3.2+	2.8+	2.6+	3.6+	2.6+	4.8+	4.6+	4.0+	2.6+	6.2+	5.0+	3.2+	3.2+	2.8+	2.4+
§ Volatile acid	1.0+	0.3+			0.2+	0.8+	1.6+	1.0+	1.0+	1.4+	0.2+	0	1.2+	2.8+	2.2+	2.2+	7.4+	7.4+
Age of seed culture in days	39	4	46	12	12	6	4	28	4	4	49	6	49		3	3	67	67
Number of sample of milk	XIII	XVIII	XXII	XXV	XXV	XXI	XXI	XXI	XXIV	XXIV	XXII	XXVII	XXII	XXIII	XXVI	XXVI	XXIII	XXIII

The sign + indicates an increase in the content of the medium.
The sign − indicates a decrease in the content of the medium.
† Milligrams per 100 cc. of the medium.
‡ Calculated as percentage of lactose.
§ Cubic centimeters of N/10 KOH required to neutralise 100 cc. of the medium.

The cultures did not have the odor of hydrogen sulphide, but on distillation of eight-day cultures large amounts of lead-blackening sulphur were given off in the distillate, which had a strong odor of hydrogen sulphide. Twenty-day cultures showed no trace of this formation of a volatile sulphide which would distinguish the culture from sterile milk. No indol or mercaptan was formed in twenty days.

Bacillus mesentericus-fuscus

The changes produced in the composition of milk by the action of *Bacillus mesentericus-fuscus* are also shown in table 2. The three analyses of eight-day cultures were made at different times, but the twenty-day cultures were incubated and analyzed on the same dates.

In eight-day cultures increases and decreases in reducing substances were observed. In twenty-day cultures the percentage of reducing substances calculated as lactose showed no change from that of the original medium. Hydrogen-ion concentration showed a decrease in eight days, but in twenty days an increase. Titratable acidity fluctuated from increase to decrease in eight-day cultures, but in twenty days showed a constant increase. The presence of acetic and butyric acid was demonstrated in the distillate, but the intensity of the color reactions appeared no greater than in sterile milk. No formation of indol, hydrogen sulphide or mercaptan was caused by the action of *Bacillus mesentericus-fuscus*.

Bacillus subtilis-viscosus

The changes in the composition of milk produced by *Bacillus subtilis-viscosus* are shown in the third column of table 2. The four analyses were made at different times.

Nitrogen as ammonia formation varied in eight-day cultures, but in twenty days was constant. Amino nitrogen increased progressively. Reducing substances calculated as lactose showed a constant decrease. Hydrogen-ion concentration showed a constant decrease, while titratable acidity increased progressively. Butyric, acetic and propionic acids were identified in the distil-

late of the culture, but the intensity of the color reactions did not distinguish the acid content of the culture from that of the original medium. No indol, hydrogen sulphide or mercaptan formation could be demonstrated.

Bacillus brevis

The changes in the composition of milk through the action of *Bacillus brevis* are given in the fourth column of table 2. The two eight-day cultures were incubated and analyzed at the same time, as were also the two twenty-day cultures.

Nitrogen as ammonia and amino nitrogen increased progressively from eight to twenty days. Reducing substances calculated as lactose showed a decrease in eight days, while in twenty days there was an increase. Hydrogen-ion concentration showed a decrease in eight days, but was unchanged from the original medium in twenty days. Titratable acidity showed a constant increase. The formation of indol through the action of *Bacillus brevis* was marked. No hydrogen sulphide or mercaptan formation could be demonstrated.

CONCLUSIONS

1. The aerobic spore-bearing bacilli investigated showed a constant and progressive increase in ammonia formation.
2. A larger and also constant and progressive increase in amino nitrogen formation accompanied the ammonia formation.
3. The actual amount of amino nitrogen present in a solution varied with the method of preparing the solution.
4. Decomposition of the proteins of milk by some of the aerobic bacteria studied proceeded beyond amino acids to indol formation.
5. Hydrogen sulphide formation was also noted in some cases.
6. Mercaptan formation was not observed in the cultures investigated.
7. A decrease in hydrogen-ion concentration accompanied an increase in titratable acidity in all cultures of aerobic spore-bearing bacteria studied, with the exception of *Bacillus albolactus*.
8. The action on milk of aerobic spore-bearing bacilli is in many respects similar to that of anaerobic bacilli.

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A MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL STUDY OF REPRESENTATIVE SPORE-FORMING ANAEROBIC BACTERIA

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From the Sheffield Laboratory of Bacteriology, Yale University

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Interest in the study of the anaerobic bacteria has markedly increased within the past few years, especially during and since the last war. It became necessary in the treatment of war wounds to know specifically the organism present so as to be able to apply appropriate serum treatment. Extensive studies in this field have been made both in this country and abroad, so that at present there is an abundance of valuable information which has contributed greatly to dispelling the chaotic state in which this phase of bacteriology had for so many years existed. Even at the present time, however, the classification of anaerobes is not established on a definite basis. A number of classification schemes have been suggested, but there has been as yet no agreement between the various workers as to the best possible system.

Realizing the need for further study the writers obtained type strains of twelve of the more common anaerobic organisms and undertook to make a more or less systematic morphological, cultural and biochemical survey. Since classification is very much simplified by the use of simple media and practical biochemical tests, particular efforts have been made to use only the more common and accepted laboratory media, and to employ the more practical methods throughout the investigation.

As a result of these studies, a broad scheme of classification based almost entirely upon the cultural and biochemical characters, with morphology as of secondary importance, became a logical

consequence. The metabolism of an organism seems to us to be of more consequence than, for example, the exact position of the spore, size of cell, or motility. These latter qualities are given an important place, however, in Hall's suggested classification (Hall, 1922). In many respects Hall's cultural results are very similar to those obtained in the present work, but the interpretation is essentially different. The writers in an earlier publication (1921) stated that gelatin liquefaction should not be considered an index of proteolysis, but simply as digestion of a derived protein, to which the term "gelatinolytic" should be applied. They followed the same course of reasoning as to the breaking down of pepton, applying the term "peptolytic" instead of "proteolytic" to such property. Miller and Reddish (1922) have gone still further and suggest that specific terms be applied to the dissolution of all kinds of protein substances.

An historical review of the subject of anaerobes will not be given here, but for such the reader is referred to the doctorate dissertation of the senior author in the Yale University library, and to the excellent reviews given by the British Medical Research Committee (1917, 1919), Hall (1922), Kendall, Day and Walker (1922), Kahn (1922), and others.

Progress in the classification of anaerobes has been seriously complicated by the numerous instances in which impure cultures were used. This fault has been largely overcome within recent years by the ready availability of type strains in national and private collections. The classification studies of recent investigators are, therefore, very definite advances over previous work in this field. In the present series, purity of strain was of first consideration, and every effort was made to obtain and maintain pure strains.

PRELIMINARY EXPERIMENTS

There are known instances in which the media or methods used for studying certain properties of the anaerobes have not been suitable, and for that reason erroneous results have been recorded. In some instances, special conditions have been claimed to be necessary whose importance is often exaggerated. For example,

it is consistently claimed that strictly alkalin media are best for the growth of anaerobes, and special efforts have been made to secure reactions in media used which ranged from −0.2 to −0.5 to phenolphthalein in the final sterilized culture media. In most cases it has been recommended that the alkali be added aseptically after sterilization, in order to obtain a definite final reaction.

A series of experiments was performed to determine the limiting hydrogen ion concentration at which representative anaerobes of this series would grow normally. One per cent glucose agar (1 per cent agar) of varying hydrogen ion concentration was used, the pH ranging from 4.8 to 8.4. It was found that *C. sporogenes* grew luxuriantly at pH 7.0, the growth being as heavy as in agar of more alkalin reaction; the growth at pH 6.6 was almost as heavy as at pH 7.0, and there were a few colonies in the glucose agar of pH 6.0. *C. tetani* grew slightly better at pH 7.4 and at pH 8.4 than at pH 7.0, but the difference was not sufficient to make it advisable to use a more alkalin reaction than pH 7.0. *Clostridium septicum*, *C. tetanoides*, *C. oedematiens*, *C. putrificum*, *C. chauvei*, and *C. Welchii* showed as good growth at pH 7.0 as at either pH 7.4 or 8.4. There was only a slight growth at pH 6.6 by *C. tetanoides*, *C. chauvei*, and *C. oedematiens*, no growth by *C. tetani* and *C. putrificum*, but good growth by *C. sporogenes*, *C. septicum*, and *C. Welchii*. It is evident, therefore, that the ordinary anaerobes can be grown successfully and with normal growth in media having a reaction of pH 7.0, which may be said to correspond in general to + 1.0 of the Fuller scale. The final pH should as a rule not be under pH 7.0 and, therefore, some allowance may be made on the alkalin side, and a final range of pH 7.0 to 7.4 aimed at. It is known that media are more subject to decomposition on sterilization when they are distinctly acid or alkalin than when they are neutral, especially when they contain carbohydrates; for this reason alone appreciable deviation from neutrality should be avoided.

Tests were made to determine the minimum amount of carbohydrate that could be safely used for fermentation tests. It was found that 0.5 per cent was the least amount that would give reliable reactions with certainty. When the sugar content was

increased to as high as 2 per cent, the reactions were not as pronounced in some cases as when it was 1 or 1.5 per cent. This was due of course to the greater acidity after sterilization when the larger amounts were used. The best combination for fermentation tests was found to be a medium consisting of the necessary food ingredients, a reaction of pH 7.0 and a sugar content of 0.5 to 1 per cent.

In order to simplify the technique as much as possible, the fermentation tests were made in deep agar tubes containing the test substances. Before adopting this method it was compared with some of the recommended procedures. The following methods were used: (1) incubation of glucose broth in an anaerobic jar, (2) covering of glucose broth with a layer of neutral mineral oil, (3) sealing of glucose broth with solid paraffin, (4) supplying glucose broth with asbestos flakes in the bottom of the tubes, (5) treating glucose broth with both asbestos flakes and a layer of oil, and (6) the use of deep glucose agar. Luxuriance of growth was used as an index of the degree of the anaerobiosis produced. The anaerobic jar in every case gave satisfactory (optimum) results and was regularly used as a basis for comparison.

Clostridium sporogenes and *C. Welchii* grew equally well under all of these conditions. *C. tetani*, *C. septicum* and *C. tetanoides* showed some growth in glucose broth covered with oil, but growth was below normal, while *C. oedematiens* and *C. putrificum* failed to grow at all with oil as the oxygen barrier. In the glucose broth under solid paraffin seal the growths of the organisms tried were very good, but even the paraffin method was found to be inferior to that of the properly used anaerobic jar. However, the deep agar culture method showed as good growth as was obtained in nutrient broth in the anaerobic jar, even in the case of *C. putrificum*, the most exacting of all of the anaerobes used.

The greater efficiency of the deep agar and solid paraffin methods over the use of oil was also shown by the employment of methylene blue in each of the tests. In the present work, the anaerobic jar and the solid paraffin seal were used for liquid media, while the fermentation tests were made in deep agar.

METHODS EMPLOYED IN THE PRESENT STUDY

Morphology

Twenty-four hour cultures in egg-meat medium (Rettger, 1906) were used. Microscopic smears were prepared from the supernatant liquid, and gently fixed in the flame. Stains were made with dilute carbol-fuchsin (1:10).

Cultural reactions

Colony form. 1. Surface. One per cent glucose agar plates, (1.6 per cent agar), previously poured and dried over night at 37°C., were streaked with a bent glass rod from young egg-meat cultures, and then incubated in an anaerobic jar at 34°C. for twenty-four hours (an incubation temperature of 34°C. was used because the paraffin seal on the anaerobic jar became soft at 37°). It was necessary to incubate *C. putrificum* ten days at this temperature in order to get good colony growth.

2. Deep colonies. Colonies in deep tubes of agar were described after three day's incubation at 37°C.

Egg-meat. Tubes of this medium were heated in a boiling water bath for fifteen minutes, cooled quickly and inoculated with sufficient material from stock egg-meat cultures to show good growth in twenty-four hours. Observations were made every day for two weeks, and after that at longer intervals; in the case of *C. putrificum* the period of observation was lengthened to six weeks.

Milk. Freshly sterilized, deep tubes of milk were inoculated with a loop of egg-meat culture, and sterile melted paraffin added to form a seal $\frac{1}{4}$ to $\frac{1}{2}$ inch thick. These were incubated at 34°C. for reasons already stated.

Broth. Plain and glucose broth, freshly sterilized, were inoculated in two sets in the same way as milk, one set being treated with solid paraffin and the other placed in the anaerobic jar. Both were incubated at 34°C.

Gelatin. Plain and glucose gelatin in deep tubes, freshly sterilized, were inoculated with the meat culture, and incubated with-

out seal at 37°C. The tubes were thoroughly chilled before observations for liquefaction were made.

Loeffler's serum. Ordinary Loeffler's serum was streaked and incubated in an anaerobic jar at 34°C. Observations were made for digestion of the serum at the end of three and ten days.

Biochemical reactions

Fermentation tests. Acid and gas production were determined in deep tubes of agar (1 per cent agar; pH 7.0), 0.5 per cent of the test substances being used. The tubes were sterilized at 15 pounds for ten minutes, quickly cooled to 45°C., and inoculated as soon as possible. Inoculations were made from diluted (1:10) twenty-four-hour plain broth cultures, 0.5 cc. being added. They were then incubated aerobically at 37°C. The following test substances were used:

Pentoses: xylose and arabinose
Hexoses: glucose, levulose, galactose, and mannose
Disaccharides: lactose, sucrose, maltose and trehalose
Trisaccharides: raffinose and melezitose
Polysaccharides: soluble starch, dextrin, glycogen, and inulin
Methyl pentose: rhamnose
Glucosides: salicin, amygdalin, esculin, and inosite
Alcohols: mannitol, glycerol, erythritol, dulcitol, and sorbitol

Plain agar tubes containing no test substance were inoculated and used as controls. Observations for gas formation were made on the third day, and again on the tenth day in the case of *C. putrificum.* At the end of this time tests were also made for acid production by adding brom thymol blue to 5 cc. of the melted agar cultures. The distance from the surface at which growth took place was measured in 19 of the test substances and in plain agar.

Glucose-consuming power. As glucose is fermented by practically all anaerobes, this sugar was used to determine the amount of carbohydrate consumed under optimum conditions. Ordinary glucose broth containing 1 per cent Bacto peptone, 0.5 per cent Armour's beef extract and 1 per cent Merck's glucose, was used. The reaction was adjusted so as to read pH 7.3 after sterilization.

The medium was filled into large tubes in 20 cc. quantities, sterilized, and cooled quickly. It was immediately inoculated with 0.5 cc. of a twenty-four-hour plain broth culture of each of the anaerobes studied. Five tubes were inoculated with each organism. One full set of the anaerobes was placed in each of five anaerobic jars, and all of the jars subjected to the same oxygen removal treatment. A set of uninoculated tubes was used for control. All were incubated at 37°C.

Benedict's method of determining glucose was employed. (See Cole, 1919.) Although the turbidity somewhat obscured the end results, duplicate determinations never revealed a difference of more than 5 per cent. However, even though 1 per cent glucose was added to the medium, the amount present, as determined by the above method, was never more than 0.8 per cent. The glucose determinations were made after the following intervals: 12 hours, 36 hours, 60 hours, 7 days, and 14 days. The hydrogen ion concentration of the cultures was also determined at these intervals.

Peptolytic property. The proteolytic property of anaerobes based upon the digestion of meat, inspissated serum, egg albumin, etc., shows merely a qualitative relationship in which the differences in degree can hardly be expressed with sufficient definiteness to be of much classification value. However, determination of the effects of growth of the organisms in pepton broth can be made more definitely quantitative. The following tests were used in the study of peptolytic action: (1) quantitative biuret, (2) combined ammonia and amino acids (Sörensen), (3) ammonia, and (4) amino-acids.

The pepton employed in all of these experiments was made as follows: 1 per cent pepton (Difco) and 0.5 per cent Armour's beef extract were dissolved in distilled water by heating in a double boiler. The reaction was adjusted to pH 7.0, and the heating continued for twenty minutes. Little precipitate came down showing that little or no food value was lost in the preparation. This is important, for, as shown by Wright (1917) and Kligeer (1917), there is often a considerable loss of food value when media are adjusted to an alkalin or an acid reaction and then

heated. Phosphates are brought down in alkalin solution and there is considerable loss of buffer action.

In the media prepared for this work, care was exercised to prevent such loss. After filtering through filter paper the broth was filled into large tubes, 35 cc. in each. Immediately after sterilizing and cooling, inoculations were made from twenty-four-hour plain broth cultures, 1 cc. being added to each tube. After arranging them in anaerobic jars, as described above for the glucose tubes, they were put under anaerobic conditions as soon as possible and incubated at 37°C. Streaks were then made from each tube upon agar slopes, for detection of contaminating aerobes. At given intervals, inoculations were made also into egg-meat media. These controls showed that no contaminations had taken place during the manipulations.

The methods of using these tests will be given in the order of their simplicity.

1. Quantitative biuret test (Vernon, 1903). This test consists simply in using standard color tubes made by adding a certain known amount of a definite solution of Witte's pepton to definite quantities of sodium hydroxide and copper sulphate solution. The standard pepton solution contained 0.25 per cent Witte's pepton and 0.25 per cent Armour's beef extract; it was filtered and sterilized in tubes. To 20 cc. of 4 per cent NaOH in Nessler's tubes, 2 cc. of 0.01 normal $CuSO_4$ were added, and, to this, amounts of the standard pepton-beef extract solution ranging from 0.2 to 1.8 cc.

In making this test 1 cc. of the unknown broth culture is added to the NaOH-$CuSO_4$ solution; after mixing thoroughly, the color formed is compared with the graded standard color tubes, and the tube which the unknown most nearly resembles in color furnishes the biuret figure.

2. Sörensen test. The test applied here is a modification of the original Sörensen test (1907), and is not claimed to be an accurate estimation of definite constituents. Both amino-acids and ammonia are titrated by this method. The test was made as follows: To 50 cc. of distilled water were added 5 cc. of the broth

culture. After treating with 1 cc. of phenolphthalein, enough dilute NaOH was used to make the medium neutral to this indicator. Five cubic centimeters of neutral formaldehyde were then added, and after stirring the mixture for about a minute, N/20 NaOH was run in until neutrality was again reached. The amount of N/20 NaOH required to neutralize 100 cc. of medium, or culture, after the first neutralization and addition of the formaldehyde, is recorded as the Sörensen figure.

3. Estimation of ammonia. Van Slyke's modification of Folin's method was used. Ten cubic centimeters of the broth culture in a long test tube were treated with 5 grams of potassium carbonate, and with a drop of caprylic alcohol to prevent foaming. Air was then drawn through, and the liberated ammonia caught in standard sulphuric acid. This acid was titrated against standard thiosulphate solution with iodine as an indicator. (See Sutton's Volumetric analysis, 8th edition, 1900.)

The difference between the number of cubic centimeters of thiosulphate solution required to neutralize the standard acid, and the number required to neutralize the same solution after ammonia has been passed into it is multiplied by the given factor, that is, the number of miligrams of nitrogen equivalent to 1 cc. thiosulphate solution.

4. Estimation of amino-nitrogen. The test cultures from which the ammonia had been expelled were diluted to 25 cc. and the amino-nitrogen determined by the micro method of Van Slyke (1913–1914, 1915). The results are expressed as milligrams of amino-nitrogen per 100 cc. This technique is so well known that a further discussion will not be given.

Pathogenicity. The pathogenicity of the anaerobes studied was determined by the injection of white mice with a 1:10 dilution of the liquid portion of seven-day-old egg-meat cultures.

EXPERIMENTAL

Type strains of the following twelve anaerobes were employed in the present investigation:

C. septicum[1]—obtained from the Lister Institute, British National collection of Type Cultures, London. Type 3. Strain George A. 2. From a case of gas gangrene developing from the surgical treatment of a healed wound. Received by the Lister Institute from Miss M. Robertson.

C. oedematiens—obtained from Dr. Gouchenour as his type strain.

C. Welchii—obtained from Lister Institute. Isolated from case of gas gangrene.

C. tertium—received from Lister Institute. Strain D 7–9—Isolated by Miss M. Robertson from material from case of gas gangrene.

C. aerofetidum—received from Lister Institute. Obtained by Miss M. Robertson from Dr. McIntosh. Strain 208.

C. sporogenes—obtained from the U. S. Army Medical School.

C. bifermentans—received from Lister Institite. Obtained by Miss M. Robertson from Dr. Weinberg. Strain H.

C. histolyticum—received from Lister Institute. Isolated from case of gas gangrene by Dr. Weinberg. Received by Lister Institute from Miss M. Robertson. Strain Weinberg.

C. tetanoides—received from Lister Institute. Isolated from septic wound.

C. chauvei—from Bureau of Animal Industry. Isolated from case of blackleg.

C. tetani—received from Lister Institute. Tullock's Type 1.

C. putrificum—isolated by Dr. Sturges, Sheffield Laboratory of Bacteriology, Yale University.

The organisms studied fall logically into five classes, or divisions, as follows:

1. Saccharolytic, but non-proteolytic, and only slightly peptolytic. Included in this group are, *C. septicum*, *C. chauvei*, and *C. oedematiens*.

2. Saccharolytic and peptolytic, but only slightly proteolytic. Examples, *C. Welchii* and *C. tertium*.

3. Saccharolytic, proteolytic and peptolytic. Examples, *C. aerofetidum*, *C. sporogenes*, *C. bifermentans*, and *C. histolyticum*.

4. Slightly saccharolytic, slightly peptolytic, and very slightly proteolytic. Examples, *C. tetanoides* and *C. tetani*.

5. Very slightly saccharolytic, but strongly proteolytic and peptolytic. *C. putrificum* is the only known member of this group.

[1] "*C. septicum*" and the other specific names ending in "um" are written thus in order to conform to the rules of nomenclature adopted by the JOURNAL.

This grouping is made only tentatively for the present in order that a more rational presentation of the following studies may be given. The reasons for placing the organisms as given above will become apparent as their characteristics are noted later. As will be seen, the term "proteolytic" as used here, denotes digestion of native protein, such as serum and egg albumin. Gelatin liquefaction is not considered an index of proteolysis, neither is the breaking down of pepton. The term, "peptolysis," is used to designate pepton decomposition. An organism may, as is clearly shown here, be gelatinolytic without being proteolytic. When meat is attacked to the extent that the particles are made finer, but the solid matter is not reduced in bulk, or when there is but slight liquefaction of coagulated serum or very little change in the appearance and bulk of casein, the organism involved is considered as being slightly proteolytic.

In the present interpretation, "saccharolytic" is used to indicate fermentation of carbohydrates with gas and acid production, while "slightly saccharolytic" is used to show some fermentation with acid, but no gas production, and slight consumption of glucose, as determined quantitatively.

Morphology, motility and colony form are, therefore, of secondary importance in such a scheme. If such a classification should be found advisible, these details could be incorporated so that complete identification of each organism could be accomplished. For the present, this rough outline will be sufficient for the purpose of grouping the anaerobes in a way that seems most logical even though they are not definitely classified.

The various properties of these anaerobes will be taken up in a comparative way, considering each factor separately. In this way, their close relation in the different groups will be more apparent.

MORPHOLOGY

Differences in the size of cells are as a rule not great enough to be of much value in identification. Outstanding peculiarities of cell form and the form, size, and position of the spore are often of much importance, particularly the location of the spore.

Clostridium septicum. This is a rather large rod, being 0.7 to 0.9 μ by 2.5 to 4 μ in twenty-four-hour egg-meat culture. Spores are oval and subterminal, and larger than the cell. They are formed readily in egg-meat medium in twenty-four hours. The rods seldom occur in chains of more than two in a chain in young cultures.

Clostridium chauvei. Varies in form from 0.7 to 0.8 μ broad to 3 to 5 μ long. In egg-meat after twenty-four hours there is no evidence of great diversity of form, except as regards length. This is contrary to descriptions often given in which pleomorphic shapes are observed. These varied forms are noted only in media, which may not be entirely favorable for normal development. Short chains are not uncommon, spores are oval and usually subterminal, and are larger than the rod. They are readily formed within twenty-four hours in egg-meat medium. However, they are never so numerous as in some of the other anaerobes.

Clostridium oedematiens. This organism is of about the same size as *C. chauvei*, being 0.7 to 0.8 μ broad by 3 to 5 μ long. Its spores are oval and are usually subterminal. The cells occur singly or in pairs, seldom in chains. Spore formation is not as abundant as in *C. sporogenes*, for example, but they occur within twenty-four hours in egg-meat medium when incubated at 37°C.

Clostridium Welchii. A rather thick rod which varies much in length, but usually measures about 1 to 1.2 μ in breadth. In twenty-four-hour meat cultures, the length varies from 2.5 to 5 μ. Longer forms, such as are often mentioned in the literature, have not been found in twenty-four-hour meat cultures. However, rods of greatly different lengths are found in young culture. The spores are quite large, usually oval and subterminal, at times central. They are readily formed in egg-meat medium within three or four days.

Clostridium tertium. This is a slender bacillus measuring 0.5 μ by 3 to 5 μ. It occurs singly or in pairs, seldom in chains. It forms oval, strictly terminal spores in twenty-four to forty-eight hours in meat medium incubated at 37°C.

Clostridium aerofetidum. Young cultures in meat medium show rather slender rods, which often occur in chains. They vary in

dimensions from 0.6 to 0.7 μ by 3 to 5 μ. Spores are formed in twenty-four to forty-eight hours, but are not abundant. They are oval and subterminal.

Clostridium sporogenes. *C. sporogenes* is a rather large rod, measuring 0.8 to 0.9 μ by 4 to 5 μ. It usually occurs singly, rarely in pairs or in chains. Spores are readily formed within twenty-four hours, and are abundant. They are oval, subterminal and much larger than the rod.

Clostridium bifermentans. Resembles *C. Welchii* in being a large rod, measuring 0.8 to 1 μ broad by 3 to 4 μ long. It often occurs in chains. Spores are oval and subterminal, and are larger than the rod. They are formed readily and become numerous in egg-meat culture within forty-eight hours after incubation.

Clostridium histolyticum. This is a rather slender bacillus 0.6 to 0.8 μ by 3 to 5 μ in twenty-four-hour egg-meat culture. It usually occurs singly or in pairs. Spores are formed in meat or egg-meat medium in from twenty-four to forty-eight hours; they are oval and subterminal.

Clostridium tetanoides. A rather long bacillus measuring 0.7 to 0.8 μ by 4 to 5 μ. It usually occurs singly, and in pairs. In three or four days round, strictly terminal spores are formed in meat culture incubated at 37°C. Both the rod and spore seem to be somewhat larger than those of the tetanus bacillus and coarser than those of *C. putrificum.* The filament formation observed in egg-meat cultures of *C. putrificum* is absent even in old cultures.

Clostridium tetani. *C. tetani* is a rather thin rod, measuring 0.6 to 0.7 μ by 3.5 to 5 μ. In old cultures there are often longer forms. In twenty-four-hour egg-meat cultures they occur mostly as individual rods, and are but rarely observed in pairs in young culture. The sporulating bacillus is the well-known drum stick, the spore being round, or almost round, and strictly terminal. Spores are not formed very readily in meat media, and in egg-meat this property seems to vary with individual strains. Three or four days are required for their appearance in egg-meat culture when incubated at 37°C. It is very difficult to obtain typical spores in some strains.

Clostridium putrificum. The morphology of *C. putrificum* might almost be said to be characteristic. At least it differs very considerably from the other common anaerobes, with the possible exception of Bienstock's "*B. paraputrificus.*" It is a long, slender rod, quite often curved, and in old cultures occurs in long filaments. It is much more slender than the other anaerobes studied, being from 0.5 to 0.7 μ by 7 to 8 μ. Forms much shorter than 7 μ are seldom seen. The spore is round and strictly terminal and very large in proportion to the rod. Spore forms are often not observed until the tenth day or after, that is, not until some digestion of the meat has taken place. No spores have been observed by us on glucose agar, nor in pure broth cultures. The spores and sporangia remain typical for a considerable time after they are formed. *C. putrificum* can be distinguished from both *C. tetanoides* and *C. tetani* on this basis. A fuller discussion of the importance of this characteristic has been given by the writers (1922 and 1923) and also by Reddish (1923).

STAINING PROPERTIES

All of the anaerobes in this series stain well with the ordinary anilin dyes. In young cultures they are all Gram-positive, but vary somewhat in the degree to which the stain is retained. For example *C. Welchii* is strongly Gram-positive, while *C. putrificum* is only weakly so. The majority of the anaerobes seem to be on the border-line between positive and negative, and in old cultures are almost always negative. There is, with the above noted exception, not sufficient difference in their deportment towards the Gram stain to call for special consideration here; furthermore, this property has already been thoroughly discussed in recent publications.

MOTILITY

Motility of an organism is a more or less variable property. At least, it is not so constant nor so easily defined as are other and more outstanding features. Because of this variability, due largely to condition of culture and to particular technique employed, this property is not emphasized in the present study.

Suffice it to say that by the plain slide and cover slip method, and the use of twenty-four-hour egg-meat cultures, the organisms group themselves as follows: Motile: *C. septicum*, *C. chauvei*, *C. tertium*, *C. aerofetidum*, *C. sporogenes*, *C. histolyticum*, *C. tetanoides*, *C. tetani* and *C. putrificum*, considerable difference being noted in the degree of motion of the different forms. Non-motile: *C. oedematiens*, *C. Welchii* and *C. bifermentans*.

Twenty-four hour egg-meat cultures were used, and observations for motility were made immediately after preparation of the slide.

COLONY FORM

Clostridium septicum. (1) Surface colonies. After three days the colonies are opaque, with dense centers and very irregular margin which takes the form of more or less tortuous off-shoots that are coarse and do not show the fine network observed in the *C. oedematiens* colonies. (2) Depth colonies. After two days at 37°C. these have a dense nucleus, with the surrounding area of the colony much thinner and a periphery surrounded with short, fine, hair-like projections. This fringed edge is not so apparent at the end of the first twenty-four hours. It is more delicate in structure than either the *C. oedematiens* or *C. chauvei* colony.

Clostridium chauvei. (1) Surface colonies. Delicate, with spreading, fine outgrowths. They are somewhat filmy and not as heavy as *C. septicum*. After three days the growths are rather tortuous, but always fine. (2) Depth colonies. About 1 mm. in diameter; there is a dense nucleus with irregular projections radiating out in matted formation.

Clostridium oedematiens. (1) Surface colonies. An opaque center with fine, transparent border, fringed with delicate off-shoots. The "nucleus" of the colony is definitely outlined and is almost round; it is finely granular, seemingly full of massed threads arranged in irregular order. (2) Depth colonies. At first lenticular; after twenty-four to forty-eight hours feathery out-growths are apparent. The outline of the colony is very much indented.

Clostridium Welchii. (1) Surface colonies. Medium-sized, white, smooth, glistening and circular, with dense center, entire edge, granular structure, and no outgrowths. (2) Depth colonies. Dense, compact and uniformly devoid of outgrowths, opaque. Some are lenticular, others round.

Clostridium tertium. (1) Surface colonies. Rather large, greyish colonies with dense center surrounded with a zone of more or less transparent nature. The latter may be in the form of outgrowths, but mostly with regular outline. There are two types, as was pointed out also by Henry (1917). The smaller, more compact type is the more common and predominant of the two. They are quite different from the surface colonies of any other anaerobe. (2) Depth colonies. Regular in outline and somewhat lenticular, with no outgrowths.

Clostridium aerofetidum. (1) Surface colonies. In general appearance resemble those of *C. Welchii*, but are coarser and more elevated. Some show denser centers surrounded by a thinner area, and this again surrounded by a thick periphery. The more characteristic colonies are more or less round, with entire edge which may be slightly wavy or undulating. The inner structure is granular and dense. (2) Depth colonies are dense and irregular, and rather small.

Clostridium sporogenes. (1) Surface colonies. Unmistakably characteristic. They have a dense center, which is definite and compact, with tortuous gnarled-root-shaped outgrowths. Detailed examination shows these to be made up of many fine filaments matted closely to give the appearance of coarseness under low power magnification. These colonies appear so early and are so characteristic that their identification in mixed cultures of anaerobes can be easily accomplished if care is taken to separate the organisms completely to insure growth in separate colonies. (2) Depth colonies. Have a dense nucleus, with densely matted hair-like periphery. Colonies are woolly, but symmetrically round.

Clostridium bifermentans. (1) Surface colonies. These are very peculiar in their conformation. They vary much in shape, being more or less amoeboid and assuming forms which differ

greatly in contour. They are rather large, irregular, dense, granular, undulating, with periphery entire and not cut up with fine projections. (2) Depth colonies. They are small and dense, and have somewhat irregular projections from a small nucleus.

Clostridium histolyticum. (1) Surface colonies. Dense, granular, with irregular periphery which is finely toothed, but which does not have definite outgrowths. The edge is thinner than the central part, but this is not clearly shown except in the higher magnifications. (2) Depth colonies. Woolly and somewhat asymmetrical, often having long, irregular projections. They may be even coral-like.

Clostridium tetanoides. (1) Surface colonies. These are dense, whitish and glistening, with wavy edge. The general contour is round, but the border is irregular, not fringed. There is a tendency to form a filmy growth over the surface of the agar; definite colonies, however, are outstanding and characteristic. (2) Depth colonies. They are small, irregular and distinctly different from the tetanus bacillus colony.

Clostridium tetani. (1) Surface colonies. The colonies are delicate, thin and spreading. They have a slightly raised nucleus, but the surrounding area is flat and in young colonies almost transparent. There are short, irregular, large, forked projections which radiate out from the central mass in an intertwining fashion. (2) Depth colonies. Are small and have a dense nucleus surrounded by fine hair-like projections; typical "woolly" colonies.

Clostridium putrificum. (1) Surface colonies. On glucose agar they do not appear until after the third day. In the present study it was found necessary to place plates under anaerobic conditions after they were examined on the third day and found to be negative. The time allowed for the second incubation was seven days, making ten days in all. Even with this prolonged incubation the colonies were found to be small, delicate, and almost transparent. The edge is irregularly dented, but not deeply so; there are no outgrowths and no apparent fringe. The structure appears to be somewhat granular, but not coarse. The aid of a hand lens is usually necessary to see the original colony clearly. It is by far the most delicate in this respect of

all the anaerobes in the present series. (2) Depth colonies. They do not appear until the third or fourth day and then they are mostly in the lower portion of the tube, although the inoculum was added at the surface of the medium. The deep agar medium is apparently better suited to rapid development, than is surface cultivation. The development of the colonies only in the deeper portion of the medium signifies the strictly anaerobic conditions that are required. The nucleus is dense, and there are fine, hair-like radiations out from the center; these projections are not long, nor are they conspicuous.

CULTURAL REACTIONS

Egg-meat

Clostridium septicum. There is a profuse evolution of gas throughout the medium in the first eight hours of incubation; this is so violent that the solid particles are usually pushed up from the bottom of the tube. The meat assumes a slightly pinkish color, and the liquid becomes more or less turbid. There is no digestion of meat, even after a month's incubation at 37°C., but there is greying of the upper portion of the medium due, presumably, to oxidation. The lower part remains pink.

Clostridium chauvei. Small gas bubbles are formed throughout the medium within the first eight hours. The supernatant liquid is slightly turbid, and the meat turned to a pinkish color. There is no digestion of the egg-meat, even after long periods of incubation. The gas bubbles leave the medium in about three days, and there is no other apparent change, except the kind of greying noted in the egg-meat cultures of *C. septicum*, which is not a cultural character, but simply the result of oxidation of the medium. Control tubes show similar greying of the upper layer of the meat.

Clostridium oedematiens. In the first twenty-four hours much gas is formed, and the medium often lifted up by the force of the gas. The liquid of the medium is slightly turbid and there is a slight reddening of the meat. At the end of two days there is evidence of a deposit of white crystals, which, however, never becomes abundant. No further change takes place in the appearance of the medium, even after prolonged incubation.

Clostridium Welchii. There is a very rapid growth with profuse evolution of gas within eight hours, and considerable clouding of the liquid and reddening of the meat. There is no digestion and no blackening of the egg-meat, even after prolonged incubation. However, there is a slight softening of the meat fibers, and a consequent closer packing of the solid particles. There is never any evidence of putrefaction.

Clostridium tertium. Gas bubbles appear in the medium after eight hours; after twenty-four hours the medium has become quite pink, or slightly red, and the supernatant liquid turbid. There is no further change, except increased reddening of the meat and clouding of the supernatant liquid. By the second day there is slight digestion, but no pronounced odor of putrefaction. Even by the eighth day there is only slight digestion of the egg-meat, with reddening and slight darkening.

Clostridium sporogenes. At the end of eight hours there is definite growth and gas production, and the meat is slightly reddened. By the twenty-fourth hour a slight odor of putrefaction is noticeable. The supernatant liquid is also slightly turbid by this time, and there is some digestion of the egg-meat. As the incubation period progresses the meat becomes at first more red, then, as digestion continues, a darkening of the medium takes place. After the second day the digestion is rapid and is accompanied with the evolution of a putrid odor. The digestion progresses to a point at which very little of the egg-meat is left, not more than a half inch remaining in the bottom of the tube; the supernatant liquid is of a dark, amber color. There is never an absolute blackening of meat, such as British workers claim occurs in beef-heart medium. This difference is probably due to differences in the media.

Clostridium bifermentans. Growth takes place within eight hours, and there is slight reddening of the meat and some gas production. By the twenty-fourth hour the supernatant liquid is turbid and the reddening of the meat has become quite apparent. After two days digestion of the egg-meat begins, but progresses only slowly. Darkening of the meat occurs soon after. By the end of the second week considerable digestion has taken place,

but to a lesser extent than in corresponding cultures of *C. sporogenes.*

Clostridium histolyticum. No gas production is evident, but there is a distinct reddening of the meat at the end of eight hours. There is some digestion by the twenty-fourth hour. This is accompanied by a putrid odor which becomes more intense after two days. The supernatant liquid becomes very turbid within the first twenty-four hours. After three or four days the egg-meat becomes darkened, and there is evident formation of tyrosine-like crystals, but since these are formed to some extent by other proteolytic anaerobes, when grown in meat or egg-meat medium, they are not considered to be particularly significant. For some reason the digestion does not progress to the same degree as does that of *C. sporogenes,* although the proteolytic action seems to be quite rapid in the early stages of growth.

Clostridium tetanoides. At the end of twenty-four hours, there is slight gas production and reddening of the meat. By the forty-eighth hour, whitish crystals, presumably of tyrosine, are observed. These increase on further incubation. This feature is more prominent in cultures of this organism than in those of any other anaerobe. After two weeks, this crystalline material has become so abundant that it takes the form of a whitish film on the surface of the medium, as well as throughout the lower portions. There is an apparent digestion of the medium, and no further change after the appearance of the crystals.

Clostridium tetani. There is no apparent change in the medium until after twenty-four hours, when there is a slight turbidity in the supernatant liquid and a faint reddening of the meat. There is usually slight evidence of gas production on the second day. After two or three weeks, there is a slight darkening of the meat at the surface. On longer incubation, this becomes very definite, but does not penetrate the medium; it does not compare in intensity with the darkening noted in the meat cultures of the strictly proteolytic anaerobes. There is no visible evidence of digestion; that is, there is no decrease in the bulk of the meat, no foul, putrefactive odor and none of the products of digestion noted in cultures of the proteolytic organisms described above. Abun-

dant growth takes place, as shown by the microscopic smears and the turbidity of the liquid. Even after prolonged incubation, there is no further change, except perhaps a slight softening of the egg-meat.

Clostridium putrificum. There is little change aside from a slight turbidity during the first three or four days; after about a week or ten days, depending upon the amount of inoculum used, there is usually a slight softening of the solid particles. The disintegration of the egg-meat is at first most apparent in the upper portion of the solid layer of the medium, but later progresses into the lower layers. Reddening of the meat has by this time become very pronounced. By the end of the second week, there is some indication of decreased bulk of the egg-meat, and distinct digestion has begun. After this stage the digestion of the meat is accompanied by the evolution of a foul odor, characteristic of putrefaction. Because of the evaporation of the liquid resulting from the digestion, there is at no time a deep layer of liquid over the still undigested portion. There is no close resemblance of the cultures to those of *C. sporogenes* or any of the other rapid putrefiers. The meat is darkened only slightly, the deep red color being dominant throughout the digestion. Only when the egg-meat has become almost entirely decomposed is there definite browning. This does not take place completely until after two months or more.

Milk

Clostridium septicum. An acid clot is formed in four or five days, but there is no digestion of the casein, even after prolonged incubation.

Clostridium chauvei. An acid clot is produced within a few days, but there is no other change.

Clostridium oedematiens. After about a week there is precipitation of the casein and later clotting, but no digestion.

Clostridium Welchii. An acid clot is formed within a few hours at 37°C. and this is followed with profuse evolution of gas. The gas production is so rapid that the coagulated casein is torn to shreds, the reaction constituting the so-called "stormy fermentation."

Clostridium tertium. There is a rapid coagulation of the casein and a slight breaking up of the curd. This action is not comparable to the stormy fermentation, so-called, of *C. Welchii*, but there is some gas and consequent rupturing of the otherwise smooth curd.

Clostridium aerofetidium. There is a precipitation of the casein, which is later slightly digested. After two weeks one-third to one-half of the casein is destroyed.

Clostridium sporogenes. After precipitation of the casein in the first twenty-four hours, its digestion follows in a very short time, and is continued until it is practically all used up, leaving a dark, amber-colored liquid.

Clostridium bifermentans. The casein is precipitated and slowly digested. In this medium, too, the action is slower than that of *C. sporogenes* in milk.

Clostridium histolyticum. The casein is precipitated early and subsequently digested.

Clostridium tetanoides. No noticeable change takes place even after several weeks of incubation.

Clostridium tetani. There is no change, except, possibly a slight precipitation of the casein.

Clostridium putrificum. There is a slow precipitation of the casein, and later almost complete digestion. The time required for this action varies with the inoculum, but more definitely with the age of the culture from which the inoculation was made. This is illustrated by the following experiment: four freshly sterilized, deep tubes of milk were inoculated with a large loop of six-weeks old egg-meat cultures of four different strains of *C. putrificum.*

Four other freshly sterilized deep tubes of milk were inoculated each with a loop of a twenty-four hour culture of these same strains; all eight tubes were sealed with solid paraffin and incubated at 37°C. The four first tubes, which were inoculated with old (digested) cultures, showed slight liquefaction at the end of twenty-four hours, and complete digestion of the casein within four days. On the other hand, the tubes inoculated with the young cultures did not show any change until the twelfth to fifteenth day, when there was evidence of digestion of the

slightly precipitated casein. One of the tubes did not show any signs of growth for four weeks, after which there was slow digestion of the casein. In all of the last four tubes, digestion took place slowly. The rapidity of the action in the first set was probably due to the preformed enzymes carried over. However, the growth would naturally be more rapid, because of the large number of organisms carried in the large loopful of old culture used for inoculum. Therefore, the time in which action in milk may be expected, will vary with the amount of inoculum and the age of culture from which the inoculation is made.

Broth

Clostridium septicum. A moderately heavy growth is shown in plain broth, and a very heavy growth in glucose broth, within the first twenty-four hours. In both there is a rapid sedimentation and consequent clearing of the medium. *Clostridium septicum* is quite similar in this respect to *C. oedematiens* and *C. chauvei.*

Clostridium chauvei. There is a moderately heavy growth, both in plain and glucose broth, but much better in the latter. This is soon precipitated, leaving the medium clear.

Clostridium oedematiens. The growth in plain broth is fairly abundant, but heavier in glucose broth. There is sedimentation of the growth almost as soon as formed, which leaves the medium clear.

Clostridium Welchii. Very abundant growth both in plain and glucose broths. There is little or no settling of the heavy growth until after one or two days, and even then the medium is not cleared to any great extent, certainly not nearly as much as in the case of the three organisms studied above.

Clostridium tertium. The growth in plain broth is only fair, but in glucose broth it is very abundant, with much sediment.

Clostridium aerofetidum. Good growth is obtained in both plain and glucose broths. After twenty-four hours there is considerable sediment, but no clearing of the medium.

Clostridium sporogenes. Growth in broth is abundant, being heavier in glucose broth than in plain broth. The turbidity is

uniform throughout the tube, and there is little settling out until about the forty-eighth hour.

Clostridium bifermentans. There is very good growth, both in plain and glucose broths. The growth is so heavy that a distinct sediment is formed very early.

Clostridium histolyticum. The growth in both plain and glucose broths is exceptionally heavy, the turbidity at the end of twenty-four hours being much greater than that of any of the other anaerobes, even *C. Welchii.* This may in fact be considered a characteristic feature, since it is so pronounced.

Clostridium tetanoides. Moderate growth takes place in plain broth, but it is much better in glucose broth. In the latter, at the end of two days, there is a very heavy turbidity which is more pronounced than that of *C. tetani* and *C. putrificum.* There is also a slight amount of gas. Some gas is formed from plain broth and plain agar, as will be noted below.

Clostridium tetani. Only moderate growth is obtained in both plain and glucose broths. No gas is formed. There is no pronounced tendency toward settling out of the growth.

Clostridium putrificum. In plain and in glucose broth the growth is slight to fair. In glucose broth, the growth is not much, if indeed any, better.

Gelatin

Clostridium septicum. Glucose gelatin is readily liquefied in twenty-four hours at 37°C., and there is sedimentation and clearing of the medium in forty-eight hours. The growth in plain gelatin is somewhat slower and the liquefaction is delayed.

Clostridium chauvei. Gelatin is liquefied rapidly, complete liquefaction taking place within twenty-four hours. It is usually somewhat delayed in plain gelatin. There is a flaky growth, which settles out rapidly, leaving the medium clear.

Clostridium oedematiens. Both plain and glucose gelatin are liquefied in from twenty-four to forty-eight hours, depending upon the amount of inoculum. The growth is flaky, as in broth, and rapidly settles to the bottom of the tube, the medium becoming clear, as in broth cultures.

Clostridium Welchii. The growth in gelatin is very profuse, and liquefaction is brought about in less than twenty-four hours in both plain and glucose gelatin.

Clostridium tertium. No liquefaction takes place, even after prolonged incubation.

Clostridium aerofetidum. There is partial liquefaction of both plain and glucose gelatin within twenty-four hours, but it is not complete until about forty-eight hours or later.

Clostridium sporogenes. Both glucose and plain gelatin are liquefied within twenty-four hours at 37°C.

Clostridium bifermentans. Glucose gelatin is liquefied in twenty-four hours, but forty-eight hours are required before there is complete liquefaction of the plain gelatin.

Clostridium histolyticum. Gelatin is quickly liquefied, the process being complete within twenty-four hours.

Clostridium tetanoides. No liquefaction of gelatin.

Clostridium tetani. There is slight liquefaction after the second or third day when a liberal amount of inoculum has been employed. With light inoculation a longer time will be required. This organism differs in this respect from the putrefiers, which all liquefy gelatin readily. The digestion is only partial at the end of four days. At the end of seven days, however, complete liquefaction has taken place.

Clostridium putrificum. Gelatin is liquefied, but the rapidity with which it is attacked varies in much the same way as noted above in the milk. That is, the age of the culture and the amount of inoculum used affect the time of liquefaction. When old cultures are used for inoculum, digestion takes place in from one to five days. When twenty-four hour egg-meat cultures are used, liquefaction is delayed considerably, taking place not before twelve to fifteen days, and often not until after three weeks. The liquefaction progresses at about the same rate in both plain and glucose gelatin.

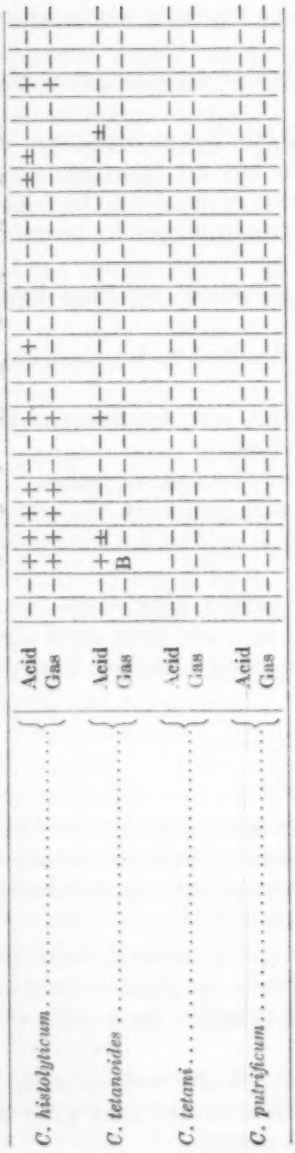
Loeffler's serum

Clostridium septicum. There is good growth, but no digestion occurs, even after twelve days at 37°C.

TABLE 1

Fermentation of test substances as shown by acid and gas production

Organism		Test substances																												
		Xylose	Arabinose	Glucose	Levulose	Galactose	Mannose	Lactose	Sucrose	Maltose	Trehalose	Raffinose	Melezitose	Sol. starch	Dextrin	Glycogen	Inulin	Rhamnose	Salicin	Amygdalin	Esculin	Inositol	Mannitol	Glycerol	Erythritol	Dulcitol	Sorbitol
C. septicum	Acid	−	+	+	+	+	+	+	−	+	+	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−
	Gas	−	+	+	+	+	+	+	−	+	+	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−
C. chauvei	Acid	−	−	+	+	+	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−
	Gas	−	−	+	+	+	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
C. oedematiens	Acid	−	−	+	+	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	Gas	−	−	+	+	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
C. Welchii	Acid	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	−	+	−	+	−	−	−
	Gas	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	−	+	−	+	−	−	−
C. tertium	Acid	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	+	−	+	+	+	+	−	−	−
	Gas	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	+	−	+	+	+	+	−	−	−
C. aerofetidum	Acid	+	−	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	+	+	±	−	−	−	−	−	−
	Gas	+	−	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	+	+	+	−	−	−	−	−	−
C. sporogenes	Acid	−	−	+	+	±	−	−	−	+	−	−	−	−	±	−	−	−	−	−	−	−	−	−	+	−	−	±
	Gas	−	−	+	+	B	−	−	−	+	−	−	−	−	B	−	−	−	−	−	−	−	−	−	+	−	−	+
C. bifermentans	Acid	−	−	+	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+
	Gas	−	−	+	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−


C. histolyticum
Acid
Gas
C. tetanoides
Acid
Gas
C. tetani
Acid
Gas
C. putrificum
Acid
Gas
B

Clostridium chauvei. Growth, but no digestion within twelve days.

Clostridium oedematiens. Growth, but no digestion, even after twelve days.

Clostridium Welchii. Abundant growth, but no digestion.

Clostridium tertium. Good growth, but no liquefaction.

Clostridium aerofetidum. Liquefaction of the serum and darkening of the resulting liquid. This is accompanied by putrefactive odors.

Clostridium sporogenes. Rapid liquefaction; resulting liquid dark and putrid.

Clostridium bifermentans. Serum is liquefied within three days, and the resulting liquid darkened.

Clostridium histolyticum. Liquefaction, and darkening of the resulting liquid, accompanied by putrid odors.

Clostridium tetanoides. Good growth, but no digestion.

Clostridium tetani. Growth, but no digestion at the end of three days; partial digestion, however, is evident by the twelfth day.

Clostridium putrificum. Growth observed after three days' incubation at 37°C., but digestion did not take place until after five days. Serum is apparently more easily attacked than the egg-meat medium.

BIOCHEMICAL REACTIONS

Fermentation tests

Clostridium septicum. Eleven of the test substances were fermented with both gas and acid production, namely arabinose, glucose, levulose, galactose, mannose, lactose, maltose, melezitose, trehalose, dextrin, and salicin. (See table 1.)

Clostridium chauvei. Seven of the test substances were fermented with acid and gas production; these are glucose, levulose, galactose, lactose, sucrose, maltose, and dextrin. Acid alone was produced from glycerol.

Clostridium oedematiens. Only 5 of the 26 test substances were fermented. Both gas and acid were formed from glucose, levulose, galactose, maltose and soluble starch.

Clostridium Welchii. Acid and gas formed from 15 of the 26 substances tested. In all cases, the amount of gas formed was considerable, and the acidity was greater than with any of the other anaerobes studied.

Clostridium tertium. Gas and acid formed from 18 of the 26 test substances.

Clostridium aerofetidum. Acid and gas were produced from 12 of the 26 agents used. The hexoses were all fermented and the disaccharides with the exception of trehalose; glycogen, salicin, esculin and amygdalin were also fermented.

Clostridium sporogenes. Only 4 of the test substances were fermented with definite acid and gas production, namely glucose, levulose, maltose and glycerol. Galactose, dextrin and sorbite gave only slight amounts of acid and gas. The saccharolytic property of this organism is, therefore, limited.

Clostridium bifermentans. Only 5 of the test substances were fermented with acid and gas production. These are glucose, levulose, mannose, maltose and glycerol. There was slight acid production from sorbitol.

Clostridium histolyticum. Glucose, levulose, galactose, mannose, maltose and glycerol were fermented with acid and gas production; slight amounts of acid were formed from melezitose, esculin and amygdalin.

Clostridium tetanoides. A small amount of gas was formed from glucose only. The amount was, however, so small that it can hardly be called positive. Acid was formed from maltose, and slight amounts from levulose and inosite.

Clostridium tetani. None of the 26 test substances were attacked with gas production, and such slight amounts of acid were produced from the others as not to be revealed by the brom-thymol-blue test. The reaction in the deep agar tubes was not reduced to pH 6.4. However, according to the results of the tests on glucose-consuming power, some acid is formed after the third day.

Clostridium putrificum. None of the test substances were fermented with demonstrable gas and acid production. At least, there was no visible gas, and tests for acid by the brom-thymol-

blue method were negative. However, as is shown below by the glucose determination method, glucose was feebly attacked. (See table 1 for fermentation reactions.)

Glucose-consuming-power

Clostridium septicum. Glucose is consumed quite rapidly, as is shown in table 2. However, the limiting hydrogen ion concentration is soon reached, and the maximum amount of sugar used is not as great as would be possible in a more highly buffered solution. Glucose is reduced from 0.727 to 0.5 per cent in fourteen days. (See tables 2 and 3.)

TABLE 2

Changes in per cent glucose in glucose broth

ORGANISM	TIME OF INCUBATION					
	Blank	12 hours	36 hours	60 hours	7 days	14 days
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C. septicum	0.727	0.625	0.500	0.500	0.500	0.500
C. chauvei	0.727	0.666	0.455	0.455	0.455	0.455
C. oedematiens	0.727	0.645	0.541	0.513	0.513	0.513
C. Welchii	0.727	0.680	0.455	0.440	0.420	0.420
C. tertium	0.727	0.526	0.465	0.465	0.445	0.445
C. aerofetidum	0.727	0.556	0.416	0.455	0.455	0.455
C. sporogenes	0.727	0.645	0.527	0.426	0.385	0.385
C. bifermentans	0.727	0.589	0.513	0.513	0.513	0.513
C. histolyticum	0.727	0.606	0.500	0.500	0.500	0.500
C. tetanoides	0.727	0.645	0.571	0.476	0.476	0.476
C. tetani	0.727	0.680	0.588	0.588	0.541	0.541
C. putrificum	0.727	0.690	0.678	0.645	0.625	0.625

Clostridium chauvei. Glucose is rapidly consumed, and to a greater extent than by *C. septicum* (0.455 per cent). The hydrogen ion concentration reached is consequently greater, the pH going as low as 4.9.

Clostridium oedematiens. Glucose is consumed rapidly, but because of the acid formed, a limiting reaction is soon reached which prevents reduction of the sugar to the fullest extent. The maximum action takes place within the first thirty-six hours, and very little sugar is consumed after this time.

Clostridium Welchii. Glucose is attacked very rapidly, the largest amount being used within the first thirty-six hours. The hydrogen ion concentration quickly drops to near the limiting point. Almost 0.3 per cent of glucose is consumed, in spite of the fact that the pH drops to 4.5 by the thirty-sixth hour.

Clostridium tertium. Glucose is consumed rapidly and the hydrogen ion concentration reached is very low.

Clostridium aerofetidum. Growth is rapid in glucose broth and there is rapid consumption of the sugar although the limiting hydrogen ion concentration is reached quickly. About 0.27 per cent glucose is lost from the medium before the pH reaches 4.7, the apparent limit of growth.

TABLE 3

Changes in hydrogen ion concentration in glucose broth

ORGANISM	TIME OF INCUBATION					
	Blank	12 hours	36 hours	60 hours	7 days	14 days
C. septicum	7.3	5.2	5.1	5.1	5.1	5.1
C. chauvei	7.3	5.4	4.9	4.9	4.9	4.9
C. oedematiens	7.3	5.2	5.1	5.1	5.1	5.1
C. Welchii	7.3	5.1	4.5	4.5	4.5	4.5
C. tertium	7.3	5.0	4.8	4.8	4.6	4.6
C. aerofetidum	7.3	4.8	4.8	4.8	4.7	4.7
C. sporogenes	7.3	5.6	5.5	5.5	5.1	5.1
C. bifermentans	7.3	5.0	5.0	5.0	5.0	5.0
C. histolyticum	7.3	5.3	5.3	5.3	5.1	5.1
C. tetanoides	7.3	5.4	5.3	5.1	5.1	5.1
C. tetani	7.3	6.9	6.4	6.4	6.0	6.0
C. putrificum	7.3	7.0	6.7	6.6	6.6	6.6

Clostridium sporogenes. Glucose is readily attacked, but not as rapidly as by *C. Welchii* or *C. tertium*, or some of the other types. Because of the alkali formation in the medium from the pepton, the limiting hydrogen ion concentration is not reached very quickly, and the consumption of the glucose continues longer than is the case with the organisms described above. In this respect the organism differs from the others. The reaction is only pH 5.5 at the end of sixty hours, and by this time 0.3 per cent glucose has been consumed.

Clostridium bifermentans. Glucose is consumed, but so rapidly that the hydrogen ion concentration is reached before alkali formation starts, and as a consequence, the amount consumed is not as great as by *C. sporogenes.*

Clostridium histolyticum. Glucose is attacked quite perceptibly, the amount consumed being the same as that used up by *C. bifermentans.*

Clostridium tetanoides. The amount of glucose consumed is considerable, certainly much greater than that used up by *C. tetani* and *C. putrificum.* This is a point of difference which is unmistakable. There is a rapid drop in per cent of glucose; by the sixtieth hour, the maximum amount has been consumed.

Clostridium tetani. Although no gas is formed from glucose some of the sugar is consumed, as is shown in tables 2 and 3. However, the amount used is small, certainly not very marked as compared with that consumed by the other anaerobes except *C. putrificum.* It is evident that the organism is not appreciably saccharolytic.

Clostridium putrificum. Glucose is attacked but slightly, and not sufficiently to be considered as indicative of definite saccharolytic property on the part of the organism. A loss of only 0.1 per cent of the glucose is recorded. Allowing for possible experimental error, the amount consumed may be even less. The ability to consume this sugar is, therefore, not an outstanding or important feature of the metabolism of this organism. The hydrogen ion changes do not keep pace with the reduction in amount of glucose, due to simultaneous production of alkali. (See tables 2 and 3.)

Peptolytic property

Clostridium septicum. This organism is only slightly peptolytic when grown in ordinary pepton broth. The decrease in the biuret figure is only 0.1 of a point, according to the standard used. The increase in Sörensen and ammonia figures is very small, as is also the change in amino-acid nitrogen. (See tables 4, 5, 6, and 7.) The amino nitrogen figure is of particular interest. There is at first a decrease, which denotes that the amino acids are being

used up by the growing organisms, and later an increase, which indicates a very slight breaking down of some of the more com-

TABLE 4

Biuret changes in plain broth

ORGANISM	TIME OF INCUBATION					
	Blank	12 hours	36 hours	60 hours	7 days	14 days
C. septicum	1.5	1.5	1.5	1.5	1.4	1.4
C. chauvei	1.5	1.5	1.5	1.5	1.4	1.4
C. oedematiens	1.5	1.5	1.5	1.5	1.3	1.3
C. Welchii	1.5	1.5	1.4	1.3	0.9	0.9
C. tertium	1.5	1.5	1.5	1.5	1.3	1.2
C. aerofetidum	1.5	1.5	1.4	1.2	0.9	0.9
C. sporogenes	1.5	1.5	1.5	1.3	1.2	0.9
C. bifermentans	1.5	1.3	1.2	1.2	1.1	0.8
C. histolyticum	1.5	1.0	0.8	0.4	0.4	0.4
C. tetanoides	1.5	1.5	1.5	1.5	1.3	1.3
C. tetani	1.5	1.5	1.5	1.5	1.4	1.4
C. putrificum	1.5	1.5	1.5	1.5	0.6	0.6

TABLE 5

Sörensen changes in plain broth

Number of cubic centimeters of N/20 NaOH required to neutralize 100 cc. of culture

ORGANISM	TIME OF INCUBATION					
	Blank	12 hours	36 hours	60 hours	7 days	14 days
C. septicum	30	30	32	36	48	51
C. chauvei	30	30	32	38	50	53
C. oedematiens	28	30	30	34	54	57
C. Welchii	28	44	58	68	88	106
C. tertium	30	30	36	40	62	71
C. aerofetidum	28	42	70	74	85	86
C. sporogenes	30	31	54	68	78	108
C. bifermentans	30	72	80	84	96	114
C. histolyticum	30	108	138	138	138	138
C. tetanoides	28	30	31	38	50	54
C. tetani	28	32	34	36	41	41
C. putrificum	28	28	30	34	80	83

plex nitrogenous substances present in the commercial pepton. The organism is to be placed, therefore, in the class of slightly peptolytic anaerobes.

Clostridium chauvei. Very closely resembles *C. septicum.* The biuret figure is the same for both, but the Sörensen, ammonia,

TABLE 6

Ammonia-nitrogen changes in plain broth. Milligrams ammonia-nitrogen per 100 cc.

ORGANISM	TIME OF INCUBATION					
	Blank	12 hours	36 hours	60 hours	7 days	14 days
C. septicum	1.301	1.95	3.25	7.67	8.45	10.50
C. chauvei	1.301	3.19	5.85	9.10	9.75	12.37
C. oedematiens	0.390	1.17	3.12	3.25	10.40	11.70
C. Welchii	0.390	7.15	11.83	13.39	14.95	15.30
C. tertium	1.301	2.34	9.75	13.39	19.76	28.73
C. aerofetidum	0.390	8.97	23.66	30.55	42.64	43.55
C. sporogenes	1.301	4.55	29.92	30.18	41.37	64.14
C. bifermentans	1.301	37.70	40.85	40.85	48.10	66.95
C. histolyticum	1.301	57.85	65.65	68.25	74.55	81.77
C. tetanoides	0.390	0.65	7.45	9.10	16.77?	14.95
C. tetani	0.390	1.43	8.71	11.05	15.34?	14.43
C. putrificum	0.390	0.52	7.15	11.05	62.40	64.94

TABLE 7

Amino-nitrogen changes in plain broth

Milligrams amino-nitrogen per 100 cc.

ORGANISM	TIME OF INCUBATION					
	Blank	12 hours	36 hours	60 hours	7 days	14 days
C. septicum	34.82	32.10	31.02	36.44	42.06	44.15
C. chauvei	34.82		30.62	35.05	46.53	47.01
C. oedematiens	29.46	29.16	31.74	32.06	50.79	47.55
C. Welchii	29.46	38.85	44.27	56.20	73.28	87.31
C. tertium	29.46	36.75	30.77	31.55	43.52	46.98
C. aerofetidum	29.46	35.00	39.07	49.53	45.49	42.99
C. sporogenes	34.82	35.73	25.50	41.00	32.68	43.36
C. bifermentans	34.82	32.58	27.94	37.04	38.60	38.98
C. histolyticum	34.82	50.11	47.50	54.99	50.70	49.75
C. tetanoides	34.82	36.10	28.54	27.44	42.54	44.91
C. tetani	29.46	29.25	23.85	25.64	34.40	34.56
C. putrificum	29.46	32.75	28.39	27.05	19.67	26.11

and amino nitrogen figures are all slightly higher for *C. chauvei.* (See tables 4, 5, 6 and 7.) The general outlines of the curves are quite similar.

Clostridium oedematiens. Pepton is attacked but sparingly. There is slightly more change in the biuret and in the ammonia and Sörensen figures than is brought about by *C. chauvei;* also in the amino acids which are perceptibly increased. (See tables 4, 5, 6, and 7.) Practically no changes take place until after sixty hours. Even these changes do not mark *C. oedematiens* as being markedly peptolytic. In fact it is only slightly so in comparison with the strictly proteolytic anaerobes.

Clostridium Welchii. The action of *C. Welchii* on commercial pepton is of particular interest, since it is non-proteolytic in the sense that it does not digest native proteins; as, for example, those of egg-meat, milk and serum, and yet vigorously breaks down the more complex products of protein digestion, namely, pepton and other biuret-given substances present in the commercial pepton. However, as noted in tables 4, 5, 6 and 7, the amount of ammonia formed is small. On the other hand, the quantity of amino-acids formed is considerable. There is a consequent increase in the Sörensen and a decrease in the biuret figure. The importance attached to the ammonia figure is again emphasized, for high ammonia production is indicative of proteolysis, or extensive breaking down of complex nitrogenous substances.

Clostridium tertium. Pepton is attacked rather sparingly, but more vigorously than by *C. septicum, C. chauvei* and *C. oedematiens.* There is a definite action on the biuret-giving substances at least, and ammonia and some amino-acid are formed. However, the increase in these products is not very appreciable, as is shown in tables 6 and 7. The decrease in biuret is proportional to the increase in ammonia, and amino-acids.

Clostridium aerofetidum. The action on pepton is not as great as in cultures of the other proteolytic anaerobes. This is to be expected, since the action on egg-meat is not markedly proteolytic, although there is unquestionable decomposition. The increase in ammonia is only moderately rapid, and the amount of ammonia produced in fourteen days is not large. Therefore, this organism is not highly peptolytic, just as it is not highly proteolytic. This correlation is borne out in many other instances to be noted later. There is a definite increase in amino-acid, but it is clearly evident

that the destruction of the ingredients of the commercial pepton do not progress to the final ammonia stage as when the strictly proteolytic organisms are employed. The greater increase in the Sörensen value accounts for both ammonia and amino-acids, and is consequently higher than either of the two. The decrease in biuret is consistent with the increase in ammonia. (See tables 4, 5, 6 and 7.)

Clostridium sporogenes. The action of *C. sporogenes* on pepton corresponds to its ability to digest native protein; pepton is readily broken down and ammonia formed in large amount. The Sörensen figure increases correspondingly. The amino-acids do not increase very much and the amounts vary more or less during the period of incubation. (See tables 4, 5, 6 and 7.) As has been previously noted, there is at first a decrease in amino nitrogen, which is due to the utilization of the amino-acids in the medium by the organism in obtaining a start in its metabolism, and later an increase, as the result of the breaking down of the pepton and simpler polypeptids.

Clostridium bifermentans. Pepton broth is vigorously attacked, even in the first twelve hours of incubation. Considerable amounts of ammonia are formed, and there is proportional increase in the Sörensen figure. The biuret figure decreases almost exactly as the ammonia increases, the correlation being particularly striking in this instance.

Clostridium histolyticum. This anaerobe is in a class by itself as regards its ability to attack pepton. The very heavy growth noted is correlated with very rapid production of ammonia from the pepton. The biuret changes are greater and more rapid than by any of the other organisms of this series. The ammonia and the Sörensen figures are far above those of the other anaerobes. The amino-acid figure does not show a constant trend, but increases, then decreases, and increases again. The curves of these figures present a picture entirely different from those of the other anaerobes studied. (See tables 4, 5, 6 and 7.)

Clostridium tetanoides. There is a greater activity in plain broth than by the members of the saccharolytic group in general, and less than by the proteolytic group. The indices of peptolysis

are more evident here than for *C. tetani*. The biuret figure decreases definitely, and the increases in ammonia and Sörensen figures are coincident with the decrease in biuret.

Clostridium tetani. The breaking down of the pepton is, in general, in keeping with the action of this organism on egg-meat and serum. There is little or no apparent change before the third day. The biuret does not show any decrease until after 60 hours, although there is a slight increase in both the ammonia and the Sörensen figures, these showing gradual change from the beginning. There is the usual drop in amino-acid nitrogen during the first few hours of growth, but there is later an increase to figures distinctly above that of the original broth. However, this increase is insignificant as compared with the other changes noted; the curves present a picture which indicates only slight peptolytic activity.

Clostridium putrificum. Pepton is attacked very little before the third day, but by the seventh day there is considerable change. By the end of a week there is a decided drop in the amount of biuret-giving substances, with a consequent increase in the ammonia and Sörensen figures. After this time, the ammonia and Sörensen figures increase only slightly. The delayed action on pepton is not surprising, because of the general tendency of the organism toward poor development in the early stages of growth. Since pepton is more easily attacked than the more complex protein of egg-meat medium, the changes noted as taking place in seven days are comparable to those apparent in meat after a longer period. Another index of the extreme peptolytic activity of the organism is the almost complete conversion of the amino-nitrogen into ammonia-nitrogen.

Pathogenicity

The following types are non-pathogenic to white mice and guinea-pigs; *C. tertium*, *C. aerofetidum*, *C. sporogenes*, *C. bifermentans*, *C. histolyticum*, *C. tetanoides*, and *C. putrificum*.

Clostridium septicum. There is edema, gas and reddening of tissues in the region of infection. Death follows usually within twenty-four hours.

Clostridium chauvei. Gas and serous exudate are formed in tissues near the site of injection. There is also a reddening of the tissues. Death follows within forty-eight hours.

Clostridium oedematiens. There is edema and reddening of the tissues in the region of injection, and death takes place within forty hours.

Clostridium Welchii. Subcutaneous tissues are pinkish, and the animals succumb if the inoculum is large. On the other hand, some strains are non-pathogenic, even in large doses.

Clostridium tetani. Mice injected into the musculature of the inner thigh show typical symptoms of tetanus within twenty-four hours. There is stiffening of the muscles, causing the animal to drag the limb and lose all use of it. Death follows within forty-eight to seventy-two hours.

GENERAL DISCUSSION

In classification studies of anaerobes the ability of the organisms to break down carbohydrate and protein substances has been considered of fundamental importance. Anaerobes are generally spoken of as proteolytic or non-proteolytic, and saccharolytic or non-saccharolytic. The term "slightly saccharolytic" is also used and is based upon whether or not the organisms attack few or many sugars, or break down any sugars to a very limited or an appreciable extent.

"Proteolysis" has been interpreted as the breaking down of substances which give, at least, some of the common tests for protein. In many cases fine distinctions have not been recognized as to whether or not all of the substances so considered were in reality protein. The outstanding instance of this is gelatin which, though it gives some of the more important tests for protein, is not a true protein, but a proteinoid. It is not included in the list of proteins given by the British Society of Physiological Chemists. In their classification they refer to it as a derivative of native protein. Most bacteriologists consider an organism proteolytic if it liquefies gelatin, even though it does not digest meat, serum-albumin, casein, etc. The breaking down of pepton has also been considered an index of proteolysis. Kendall and

his co-workers have based their claims regarding the protein-sparing action of carbohydrates on the sparing of pepton in sugar media. This is technically an incorrect use of the term and must lead to confusion.

There is urgent need of a more concrete definition of the terms "saccharolytic" and "proteolytic." Egg albumin and serum albumin are native proteins. If an organism attacks these with the elaboration of products characteristic of such action, it should be considered proteolytic. Destruction of substances which are not native protein, should not come in the same category. In the case of gelatin, liquefaction may be brought about by enzymes known as gelatinases, and this action may be entirely independent of proteolytic activity. This fact has been brought out by Berman and Rettger (1918) in their studies on the utilization of protein and non-protein nitrogen by the members of the colon-typhoid group. They state specifically that "the ability of an organism to liquefy gelatin is no sure indication of its proteolytic properties." It is therefore thought entirely reasonable to define the term proteolysis to mean the digestion or decomposition of native protein.

The number of carbohydrates attacked and the degree to which glucose is consumed have both been considered in determining saccharolytic property. The qualitative testing for acid and gas is in keeping with general practice, while the determination of glucose-consuming power is an attempt to substitute quantitative for qualitative criteria, or at least support them by accurately-determined quantitative data. The combination of the two makes for definiteness and certainty, and for that reason should prove a distinct aid in classification studies.

No considerable emphasis is placed here upon slight differences in the fermentation of test substances by the various organisms. Only where the differences were marked will they prove of classification value. For example, *C. Welchii* can be distinguished from *C. septicum*, *C. chauvei* and *C. oedematiens* by the large number of sugars it ferments. *C. tertium* ferments even more sugars than *C. Welchii*. On the other hand, *C. sporogenes*, and *C. histolyticum* cannot be so surely distinguished from *C. bifermentans* on this basis because the differences are slight, when purely fermen-

tative reactions are considered. Although *C. tetanoides* produces acid from a few substances, it is unsafe to make a distinction between it and *C. tetani* or *C. putrificum* on this basis. Therefore, it is not always safe to identify anaerobes according to fermentation tests and it is inconsistent with scientific accuracy to place undue emphasis upon factors which are apt to be inconstant. Not only are individual strains of the same species apt to vary in this respect, but even type strains may do so, especially after having been carried on artificial media for a long time.

The form and position of the spore and the colony form are of great importance in the identification of the anaerobes. Certain of the cultural characters are quite similar for certain organisms. For example, the appearance of the meat, milk, broth, gelatin and serum cultures of *C. septicum*, *C. chauvei*, and *C. oedematiens* are very much alike. These organisms vary somewhat in their action on glucose and peptone, but these differences are not sufficient to be of classification value. The sources of these three organisms, their specific pathogenic properties and their serological differences are more important means of differentiation than are the cultural and biochemical tests. The fermentation tests of these three types may be of more or less definite help in identification, but these must be secondary to the other properties given.

Greater differences are noted in the cultural and biochemical reactions of the rest of the anaerobes studied. *C. Welchii* shows marked differences in many ways from all of the other anaerobes. Colony form is characteristic, as is also the milk reaction; its action on glucose is more vigorous than that of the other organisms studied; it forms larger amounts of amino-acid from pepton, or rather the large amount of amino-acid produced is not further reduced to ammonia, as is the case with other anaerobes which form large amounts in the process of protein-digestion. The ammonia figure, however, is only slightly above that of the first three organisms listed above. This species is, therefore, intermediary between the non-proteolytic and proteolytic, or more strictly, between the peptolytic and non-peptolytic type. *C. tertium* shows still less amino-acid and slightly more ammonia, being

therefore, more peptolytic than *C. Welchii*. This seems to be true even though the biuret changes are not so great as in the case of *C. Welchii*. Figures representing changes in pepton by *C. aerofetidum, C. sporogenes, C. bifermentans and C. histolyticum* show distinct and active peptolytic activity. These are differences which may be of value in classification, and are here emphasized as offering great possibilities in this connection.

C. tetanoides and *C. tetani* are only slightly peptolytic; they are only slightly saccharolytic, also. According to the brom-thymol-blue tests, both *C. tetani* and *C. putrificum* may be considered non-saccharolytic. The more accurate quantitative tests, however, show slight action on glucose.

Figures showing deportment of *C. putrificum* towards glucose and pepton are characteristic. The most outstanding properties of this peculiar anaerobe are best illustrated by these data. In case of doubt as to the identity of any given anaerobe which resembles this organism, such tests can be relied upon to establish definitely whether or not it is *C. putrificum*. The addition of these quantitative tests is of more value than might at first appear and it is hoped that considerably greater use of biochemical tests in general will be made in the future. It is by means of such definite data that progress in classification must advance.

Wolf and Harris (1918–1919) report 12.3 mgm. of ammonia N formed by *C. septicum* in tryptic broth in forty-eight hours. This figure is higher than that found in the ordinary broth used in the present experiments for the same length of time, but is quite near the figure representing the amount formed in 14 days, which is 9.2 mgm. The amount of amino-acids formed by *C. septicum* is not considerable, as is shown above. This is in keeping with results reported by Kendall and others. The writer found 9.33 mgm. amino N at the end of fourteen days, and Kendall found from 0.7 mgm. to 9 mgm. in the same length of time. *C. chauvei* is quite similar to *C. septicum* both in glucose-consuming power and in peptolytic property, as is shown in the accompanying tables.

Wolf (1920) claims that *C. oedematiens* is proteolytic because ammonia is formed from protein media. Our results show such

a small amount of ammonia formed as not to be indicative of pronounced peptolysis. Wolf reported 17.8 mgm. ammonia formed in one hundred sixty-eight hours in tryptic broth, while our studies show only 11.07 mgm. ammonia N in pepton broth in fourteen days. For *C. Welchii*, Kendall, Day and Walker (1922) obtained figures ranging from 7.7 to 18.9 mgm. ammonia N per 100 cc. in fourteen days, while we obtained 14.91 mgm. in the same length of time in the same kind of medium. Kendall and his associates obtained very low figures for ammonia formed by *C. tertium;* they found only 0.7 mgm. produced per 100 cc., while the writers found 27.43 mgm. in fourteen days. It is evident that very different strains were used. The ammonia figures for *C. aerofetidum* obtained here agree somewhat with those of Kendall. His figures show from 51.8 to 66.5 mgm. ammonia compared to 43.16 obtained by the writers. There is much discrepancy between the figures representing ammonia formed by *C. sporogenes* as given by Wolf and Harris (1916–1917) and by Kendall, Day and Walker (1922), and those given in the present work. Wolf and Harris report 77 mgm. ammonia in 7 days; the writers obtained 40.07 mgm. in the same period, while Kendall and his co-workers found 84 and 106.4 mgm. as their figures for this period. With *C. bifermentans* closer comparison is shown between our figures and those of Kendall. His figures range from 54.2 mgm. to 60.9 mgm. in fourteen days as compared to 65.65 mgm. reported here.

Kendall's data on *C. histolyticum* in pepton broth show lower ammonia figures than those obtained in the present work. We found 80.47 mgm. in fourteen days, as compared to 46.9 to 60.9 mgm. obtained by Kendall. Our ammonia nitrogen determination (14.5 mgm. in fourteen days) closely approximated that of 13.3 mgm. by Kendall, as did also the ammonia nitrogen figures for *C. tetani* (Kendall's 9.8 to 14.7; authors' 14.04 mgm.).

Our biochemical data on *C. putrificum* are not to be compared with those of other workers, both English and American. It is evident that our strains are distinctly different from those used by the other investigators. A full discussion of *C. putrificum* will be found in recent papers by the writers, in which are described the differences between this organism and *C. sporogenes*, which

is the closest approach to the type of organism used by both English and American investigators. Our strains correspond to descriptions given Bienstock (1884, 1889, 1901, and 1906), Distaso (1911) and others, and it seems quite certain that they are true to the type first described by Bienstock. Furthermore, *C. putrificum* has been confirmed as a distinct species by the writers in recent publications.

SUMMARY AND CONCLUSIONS

Morphological studies were conducted on anaerobes under conditions which are most favorable for growth, and in which typical forms were consistently present.

Studies of both surface and depth colonies were also made, since we believe that colony morphology is of considerable importance in the identification and classification of the organisms.

Cultural and biochemical studies were pursued on the simplest media and by the use of the simplest available technique.

The fermentation experiments were carried out on 26 test substances in deep agar tubes. This method afforded definite results and was found to be convenient and satisfactory.

The glucose-consuming power of the anaerobes was estimated by the use of quantitative chemical methods.

As a further aid in classification, the peptolytic property of the organisms was determined in plain pepton broth. The quantitative biuret test, Sörensen test, estimation of ammonia by the iodine-thiosulphate method, and the determination of amino-nitrogen by the van Slyke method were employed in this study.

Pathogenic property was tested on white mice.

The data in this investigation are given in such a way that they can be used for identification or classification purposes, but no actual attempt at classification is made; a tentative grouping is given for convenience in presenting the material in logical order.

As a result of these studies, the following conclusions are drawn:

For consistent results, all morphological, cultural and biochemical studies should be made under exactly the same conditions, in the same kinds of media, and incubated for the same length of time and at the same temperature.

Liquefaction of gelatin should not be used as an index of proteolysis.

Native proteins, such as serum-albumin and egg albumin, should be used in the tests for proteolysis.

For classification purposes, the position and shape of the spore, colony form, particularly surface, action on native protein, extent to which glucose is consumed, as well as the kind and number of substances fermented, peptolytic property, and pathogenicity are of the most importance.

Differences, in liquefaction of gelatin, in the fermentation of particular test substances, and in motility, are of relatively little value as criteria for classification.

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CORRELATION OF CITRATE UTILIZATION BY MEMBERS OF THE COLON-AEROGENES GROUP WITH OTHER DIFFERENTIAL CHARACTERISTICS AND WITH HABITAT.

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In a previous study of the utilization of salts of various organic acids it was found that two sections of the colon-aerogenes group were clearly distinguished by the use of a chemically definite medium containing sodium, potassium, or ammonium citrate as the only source of carbon (Koser, 1923). The methyl red positive, Voges-Proskauer negative cultures of fecal origin —typical *Bact. coli*—were incapable of utilizing the citrate radical as a source of carbon and consequently did not develop, while the methyl red negative, Voges-Proskauer positive cultures—*Bact. aerogenes* and allies —readily attacked the citrates. Throughout the previous investigation the testing of a number of different organic acids necessitated the employment of a limited number of colon-aerogenes strains. In view of the different habitats in which members of the colon-aerogenes group have been found in nature and of the recent interest attached to these types from a sanitary standpoint, it was deemed advisable to continue the study of citrate utilization by employing a larger number of strains from as many different sources as possible.

In the present investigation the ability to utilize the citrate radical has been compared with the source of the cultures in nature, with glucose metabolism in a buffered peptone glucose medium, as brought out by the well-known methyl red and Voges-Proskauer tests, and with the ability to utilize the nitro-

gen in the purin ring of uric acid. All non-spore-forming, Gram-negative rods fermenting glucose and lactose with the production of acid and gas and capable of growing aerobically were considered as members of the colon-aerogenes group. All of the strains used in the present study were isolated by the writer and consequently the history, including the source of original isolation, was definitely known. The cultures were obtained from locations which represented the opposite extremes of sanitary standards. One hundred and eighteen cultures were obtained from fecal specimens, while seventy-two cultures were isolated from soils as far removed from any chance of pollution as possible.

The fecal cultures were secured from man as well as from a number of domestic and laboratory animals. The method of isolation consisted in emulsifying part of the fecal specimen in sterile water and streaking a loopful of the resultant thick suspension over Endo plates. After eighteen to twenty-four hours incubation several colon-like colonies, never more than four or five from one specimen, were transferred to lactose broth and to plain agar slants. If the cultural properties in these media showed the organisms to belong to the colon-aerogenes group the process of fishing from well-isolated colonies on an Endo plate was repeated before the cultures were added to the stock collection. As a rule, only typical, flat, dark-red colonies with a metallic luster appeared on Endo plates streaked from the fecal specimens. Occasionally, however, in addition to this type of colony, lactose fermenters were found which produced raised pink colonies, frequently more or less mucoid. Whenever these appeared, one or two representative colonies, depending upon the proportion present on the plate, were also isolated as described and added to the stock collection for future tests. A few of the fecal specimens from laboratory animals, notably those from guinea-pigs, contained so few colon organisms that no colonies were obtained by direct streaking of emulsions on Endo plates. In these cases the fecal samples were added to lactose broth and after twenty-four hours incubation the resultant cultures were streaked on Endo plates. Representa-

tives of the colon group were usually obtained after this enrichment. After such a procedure, however, only one colony was picked as representative of each fecal specimen, unless more than one type of colon-like colony appeared on the plates.

Every effort was made to secure soil samples from locations where the chance of human pollution would be very slight. Most of the samples were collected in Virginia and Maryland within a radius of twenty miles of the District of Columbia and were taken from wooded hilltops, usually where the underbrush was very dense and where the possibility of any chance human pollution seemed to be very improbable. A few soil samples were taken from the South Mountain range of the Alleghanies in eastern Pennsylvania. These specimens were collected on mountain summits at a considerable distance from roads or trails. The nearest habitations, several small farmhouses, were a mile or more distant in a valley. While the possibility of pollution by small wild animals or birds could not be entirely excluded from any of these locations, nevertheless, it is believed that organisms obtained from such sources may justly be regarded as representative of a normal unpolluted habitat.

The procedure followed when collecting the soil samples consisted in first scraping away the surface layer of decaying leaves, etc., and then taking the sample from the surface soil, or from a depth of not more than 1 to 2 inches below the surface. A flat sterile spatula or other flat instrument was used for transferring the soil to sterile, wide-mouth glass bottles which were used as containers for transporting the specimens to the laboratory. The glass stoppers were protected by a layer of cotton covered with several thicknesses of gauze.

The isolation of colon-aerogenes organisms from soil was first attempted by preparing dilutions of the soil samples and plating directly from them. Owing to the scarcity of these types in the soils studied, however, this method failed to detect many organisms which were found upon the enrichment of larger portions of the samples in flasks of lactose broth. The method of isolation finally adopted was to add 10 to 15 grams of each soil sample to 75 cc. of 0.5 per cent lactose broth con-

tained in 200 cc. Erlenmeyer flasks. These were incubated at 30°C. and from the resultant cultures Endo plates were streaked after twenty-four hours incubation and sometimes again after four or five days.

The necessity for repeated examination of the soil cultures and of the Endo plates made from them was brought out by the occasional finding of colon group organisms which exhibited a delayed fermentation of lactose. While such organisms would be overlooked by the ordinary methods of examination, it was felt that they should be included in any study of the colon-aerogenes group and consequently the usual procedures were modified to a certain extent in order to detect them. When fishing colonies from Endo plates the selection was not limited to the usual red or pink colon colonies, but all forms were included which in any way resembled the type of colony formed by the colon group, whether showing the red coloration or not. These were transferred to 0.5 per cent lactose broth tubes and held for fourteen days or more at 30°C. All cultures thus found to be capable of fermenting lactose were streaked again over Endo plates and reisolated before adding them to the stock collection. By following this procedure several colon group cultures which exhibited a delayed fermentation of lactose were obtained. These cultures formed small colorless colonies on Endo plates after twenty-four hours at 30°C., but when held for several days the colonies became much larger, frequently assuming a deep pink mucoid appearance. When transferred to lactose broth there was no evidence of fermentation until after three or four days or occasionally longer. The hydrogen-ion concentration attained by these cultures in lactose broth was never as great as that of the fecal coli strains, but on the other hand the evolution of gas was very pronounced after the fermentation had begun. While members of the colon group which attack lactose tardily have seldom been reported, owing no doubt to the methods of examination employed, it is evident that they constitute an appreciable part of the flora of the soils studied in the present investigation, for of the 72 colon group cultures from soil, 14 exhibited a delayed lactose fermentation. Most

of these produced acid and gas in a 0.5 per cent lactose broth within three or four days at 30°C. although a few showed no evidence of fermentation until the seventh or eighth day. Colon group organisms showing a somewhat similar delayed fermentation of lactose have been encountered in certain foodstuffs (Bronfenbrenner and Davis, 1918; Koser, 1920).

Where lactose fermenters were found to be present in a soil culture after enrichment, only one strain from each sample was kept for further study unless distinct differences in the Endo colony, growth, etc., were apparent. In these cases one representative of each type was isolated and retained. Seventy soil specimens were examined and from them 72 cultures of the colon-aerogenes group were obtained. Seventeen soil samples gave negative results, 36 yielded one culture each, 15 two cultures each and from each of 2 samples three strains were obtained. In those cases where two or more different cultures were found in the same sample, subsequent investigation showed that the cultures frequently belonged to different sections of the group as brought out by the differential tests.

Descriptions of the media and procedures employed in the present investigation are given below.

Citrate medium. For the test of citrate utilization a chemically definite medium containing certain inorganic salts, an inorganic source of nitrogen, and a citrate as the only source of carbon was employed. Several different combinations may be used. Two of the most convenient are given here. [1] 5 grams NaCl, 0.2 gram $MgSO_4$, 1 gram $(NH_4)\ H_2PO_4$, 1 gram K_2HPO_4, and 2 grams sodium citrate (2.77 grams sodium citrate. $5\frac{1}{2}\ H_2O$) in 1000 cc. of distilled water. [2] 1.5 grams $Na\ (NH_4)\ HPO_4 + 4\ H_2O$ (microcosmic salt), 1 gram KH_2PO_4, 0.2 gram $MgSO_4$, and 2 grams sodium citrate in 1000 cc. of distilled water. The hydrogen-ion concentration of both of these media is about 6.7 to 6.9. Potassium citrate may be substituted for the sodium citrate. Ammonium citrate constitutes a convenient combination of a source of nitrogen with the citrate radical, but has the disadvantage of being a very unstable salt.

The above media were tubed in 5 to 8 cc. quantities, sterilized in the autoclave and inoculated directly from plain agar slants of the colon-aerogenes cultures. The temperature of incubation was 30°C. Observations of growth, as shown by visible turbidity, were made at intervals of one, two, three, four, and seven days and where negative results were obtained after two and three weeks.

Uric acid medium. The composition of the uric acid medium was the same as that previously described (Koser, 1918). Since development in this medium depends upon the presence of available nitrogen some care must be exercised to exclude free ammonia in so far as possible. Also the medium should be inoculated lightly from young agar cultures in order to avoid carrying over any superfluous quantity of dead cells, enzymes, etc. In one set of experiments 0.2 per cent glucose was substituted for the 3 per cent glycerol as the source of carbon, since it was though that perhaps glycerol might not be readily utilized by some of the colon group cultures. However, similar results were secured in both cases. The temperature of incubation and the method of observing development were the same as those described for the citrate medium.

Methyl red and Voges-Proskauer tests. Cultures for these tests were grown for four days at 30°C. in the medium recommended by Clark and Lubs (1915)—0.5 per cent Witte pepton, 0.5 per cent dipotassium phosphate and 0.5 per cent glucose. Care should be taken to secure a dipotassium phosphate of known purity. A dilute solution of K_2HPO_4 should give a deep red to phenol red or a light pink to phenolphthalein. Details of the technic of these tests given in the American Public Health Association Standard Methods (1920) were followed with one exception. The Voges-Proskauer tests, after addition of the 10 per cent potassium hydroxide solution, were incubated at 37°C. for five or six hours and after recording the results at this time they were held overnight at room temperature and again examined to detect any additional positive tests.

Every culture was run through each of the differential tests—methyl red, Voges-Proskauer, citrate and uric acid media—several

times to determine whether there would be any variation in these tests when repeated at different intervals. As will be shown later, a few cultures showed variation in the results of the methyl red and Voges-Proskauer tests. These cultures were tested repeatedly—ten to fifteen times or occasionally more. In summarizing the work in tables 1 and 2, all cultures are recorded as definitely positive or negative in the four differential tests only if the results were consistent upon several different occasions. The production of visible growth in the synthetic media is given at the expiration of three days, since with one or two exceptions all cultures capable of development in these media produced a definite turbidity within twenty-four or forty-eight hours, and therefore the three-day interval appears adequate for routine purposes.

Table 1 presents the results obtained with the colon group cultures from fecal specimens. It is evident that growth in the citrate and uric acid media correlates almost perfectly with the methyl red and Voges-Proskauer tests. Of 109 methyl red positive, Voges-Proskauer negative cultures from human and animal feces, 107 failed to develop in the citrate medium while 108 gave negative results in the uric acid medium. Of the two methyl red positive cultures which utilized citrate, one could not be considered as a typical *Bact. coli* since its cultural and fermentative properties resembled *Bact. aerogenes*. Also, this strain produced a luxuriant raised pink colony on Endo plates and developed readily in the uric acid medium. One additional culture exhibited a delayed utilization of citrate, producing visible turbidity regularly after five to seven days. This was the only instance in which a result of this kind was encountered. Nine cultures of the aerogenes type were all capable of rapid and luxuriant growth in both the uric acid and citrate media.

The soil cultures are shown in table 2. Here it will be seen that all the aerogenes-cloacae types developed readily in the citrate medium and that this property was correlated with alkalinity to methyl red, a positive Voges-Proskauer reaction and in most cases with growth in the uric acid medium. A

TABLE 1

Cultures from fecal specimens

SOURCE OF CULTURES	NUMBER OF CULTURES	PEPTONE, GLUCOSE, K_2HPO_4 (4 DAYS AT 30°C.)		GROWTH AFTER 3 DAYS AT 30°C.		REMARKS	REFERENCES TO TABLE 3
		Methyl red	Voges-Proskauer	In citrate medium	In uric acid medium		
Typical fecal coli							
Human	30	+	0	0	0		
Sheep	8	+	0	0	0		
Fox	1	+	0	0	0		
Dog	13	+	0	0	0		
	1	+	0	0*	0		A
Cat	8	+	0	0	0		
Rabbit	26	+	0	0	0		
Rat	13	+	0	0	0		
Guinea-pig	7	+	0	0	0		
	1	+	0	+	0	Typical *Bact. coli.*	
	108						
Atypical							
Rabbit	1	+	0	+	+	Endo colonies and cultural properties similar to aerogenes type	B

Fecal aerogenes

Rabbit	3	0	+	+	+		
Guinea-pig	3	0	+	+	+		
	2	0	Weak + or 0	+	+	Cultural properties resembling *Bact. aerogenes*	
Human	1	0	Weak + or 0	+	+		
	9						
Total	118	+ = 109 0 = 9	0 = 109 + = 6 Var. + = 3	0 = 107 + = 11	0 = 108 + = 10		

Of 118 fecal cultures:

107 (90.7 per cent) failed to develop in the citrate medium

108 (91.5 per cent) failed to develop in the uric acid medium

109 (92.4 per cent) were methyl red positive and Voges-Proskauer negative

* Delayed in growth in citrate medium, becoming + regularly after five to seven days.

TABLE 2

Soil cultures

NUMBER OF CULTURES	PEPTON GLUCOSE K_2HPO_4 (4 DAYS AT 30°C.		GROWTH AFTER 3 DAYS AT 30°C. IN		REMARKS	REFERENCE TO TABLE 3
	Methyl red	Voges-Proskauer	Citrate medium	Uric acid medium		
36	0	+	+	0 or ? = 7 s.l. + = 3 + = 26	Typical aerogenes-cloacae types	
1	0	Weak + or 0	+	Sl. +	Cultural and fermentative properties resembling aerogenes-cloacae types	
2	Neutral tints	+	+	+		
8	Var.*	Var.*	+	+	Cultural and fermentative properties resembling aerogenes-cloacae types	C1 to C8
23	+	0	+	0 or ? = 20 Sl. + = 2 + = 1	(See table 3 for details)	D1 to D23
2	+	0	0	0	Resembling typical *Bact. coli*.	E1, E2
72	0 = 37 ? or var. = 10 + = 25	+ = 38 Var. = 9 0 = 25	+ = 70 0 = 2	+ or s.l. + = 43 0 or ? = 29		

*Of 72 soil cultures:

70 (97.2 per cent) utilized citrate and in this respect were distinct from the fecal Bact. coli type.

43 (59.7 per cent) developed in the uric acid medium.

38 (52.8 per cent) were Voges-Proskauer positive and 37 (51.4 per cent) were alkaline to methyl red, consistently and upon repeated tests.

*8 (11.1 per cent) exhibited a change from the methyl red positive, Voges-Proskauer negative type when first isolated to a methyl red negative, Voges-Proskauer positive reaction upon later cultivation (see text for details).

25 (34.7 per cent) were consistently methyl red positive and Voges-Proskauer negative.

number of cultures which gave irregular or variable results with the methyl red and Voges-Proskauer tests were all capable of utilizing both citrate and uric acid. The general cultural and fermentative properties of these organisms resembled those of the aeorgenes-cloacae section of the group. In addition to these types there were 25 soil cultures which were consistently methyl red positive and Voges-Proskauer negative. Twenty-three of these failed to show the usual correlation between the differential tests, for they were methyl red positive and also developed readily in the citrate medium. The ability to utilize citrate exhibited by the methyl red positive organisms from soil is noteworthy and presents a decided contrast to the negative results given by the methyl red positive strains of fecal origin shown in table 1.

The large number of methyl red positive colon group organisms from soils which were considered free from human pollution was quite surprising. They constituted 34.7 per cent of the total number of soil cultures, a higher proportion than usually reported. Johnson and Levine (1917) found that 19.7 per cent of the lactose fermenters from field and garden soils belonged to the coli section. Burton and Rettger (1917) isolated 193 colon-aerogenes cultures from soil, leaves, twigs, etc., and found only 36, or 18.7 per cent, which resembled *Bact. coli*. Chen and Rettger (1920) reported that 20 of 467 soil strains, or 4.3 per cent, belonged to the methyl red positive type. In a study of colon-like bacteria found on grains, Rogers, Clark, and Evans (1915) encountered a few cultures which gave a CO_2:H_2 ratio similar to that of the fecal *Bact. coli* but which differed from the characteristic fecal cultures in that they formed a light yellow pigment.

To determine if possible to which section of the group the several variable and irregular cultures might be related, their cultural and fermentative properties were further studied. The detailed results secured with the most perplexing organisms are given in table 3. It should be noted here that all irregular cultures were replated and reisolated several times in an effort to eliminate contaminants as the cause of the irregularity. No contaminating organisms could be found and the cultural charac-

TABLE 3

Irregular and variable cultures

REFERENCE FROM TABLES 1 AND 2	FERMENTATION OF						GELATIN LIQUEFACTION	INDOL	ENDO COLONIES
	Lactose	Salicin	Sucrose	Raffinose	Dextrin	Adonitol			
Fecal origin									
A	+	+	0	0	0	0	0	+	Coli-like
B	+	+	+	+	+	+	0	0	Large mucoid, pink
Soil origin									
C1	+	+	+	+	0	+	0	0	Large pink
C2	+	+	+	+	0	+	0	0	Large mucoid, pink
C3	+	+	+	+	0	+	0	0	Large pink
C4	+	+	+	+	0	+	+	0	Large mucoid, pink
C5	+	+	+	+	0	+	0	0	Large pink
C6	+	+	+	+	0	+	0	0	Large pink
C7	+	+	+	+	0	+	0	0	Large pink
C8	+	+	+	+	0	+	0	0	Large pink
D1	+S	+	+	+	0	0	+	0	
D2	+	+	+	+	0	0	0	+	With the exception of the slow lactose fermenters, colonies were usually quite similar to those of typical fecal *Bact. coli*. Flat, deep red and occasionally showing metallic lustre
D3	+	+	+	+	0	0	0	+	
D4	+	+	+	+	0	0	0	+	
D5	+	+	+	+	0	0	0	+	
D6	+	+	+	+	+	0	0	+	
D7	+	0	0	0	0	0	0	0	
D8	+S	0	0	0	0	0	0	0	
D9	+	+	0	0	0	0	0	0	
D10	+	0	0	0	0	0	0	0	
D11	+	0	0	+	0	0	+	0	
D12	+	+	+	+	0	0	+	+	
D13	+S	+	0	0	0	0	+	0	
D14	+	0	0	0	0	0	+	0	
D15	+	0	0	0	0	0	+	0	
D16	+	0	0	0	0	0	0	0	
D17	+	0	+	+	0	0	0	0	
D18	+S	+	0	0	0	0	0	0	
D19	+S	+	0	0	0	0	0	0	
D20	+S	+	0	0	0	0	0	0	
D21	+	0	0	0	0	0	0	0	
D22	+	+	+	+	0	0	0	+	
D23	+	0	0	0	0	0	0	0	
E1	+	+	0	0	0	0	0	+	Coli-like
E2	+	0	0	0	0	0	0	+	Coli-like

Delayed lactose fermentation is designated as +S.

teristics exhibited by the newly isolated cultures were the same as those of the old cultures.

Among the irregular cultures several appear as especially noteworthy. Thus, one of the strains obtained from rabbit feces (B—tables 1 and 3) shows the cultural and fermentative properties of *Bact. aerogenes*, although the results of the methyl red and Voges-Proskauer tests were regularly those of *Bact. coli*. This culture produced a luxuriant growth in both citrate and uric acid media. Eight cultures (C1 to C8, tables 2 and 3) from soil exhibited an interesting reversion in the methyl red and Voges-Proskauer tests. When tested immediately after isolation these cultures were all methyl red positive and Voges-Proskauer negative upon incubation at 30°C. for four days. After cultivation upon agar slants in the laboratory for a month or more they became methyl red negative and Voges-Proskauer positive. This change was gradual and not abrupt. Repeated tests during this time showed a transition period during which the culture would exhibit a neutral tint to methyl red with the Voges-Proskauer test negative or questionable after four days at 30°C., whereas when tested after seven days at 30°C. the reaction would be of a more definite methyl red negative, Voges-Proskauer positive type. Finally, after further cultivation, these cultures always gave a definite methyl red negative, Voges-Proskauer positive test after four days at 30°C. and then appeared to be fixed since further variation was never observed. During the transition period, when it became evident that the methyl red and Voges-Proskauer tests were not checking with previous tests, these cultures were replated upon Endo medium and well-isolated colonies were again transferred to agar slants. All of the colonies appearing upon the Endo plates presented the same appearance, with no evidence of contaminations. The old cultures were retained along with the newly isolated ones and thereafter the two sets from each of the eight strains were tested side by side in the glucose, pepton, dipotassium phosphate medium, in citrate medium and in uric acid medium. No difference was apparent in the behavior toward citrate and uric acid. Results with the methyl red and Voges-Proskauer tests

showed no difference between the newly isolated and the older cultures since both exhibited the transition to the methyl red negative, Voges-Proskauer positive type of reaction.

While it is realized that contaminations cannot be positively excluded in these cases, nevertheless it is believed that the change in the tests as observed cannot be accounted for in this manner. It seems probable that as a result of laboratory cultivation the secondary or alkaline fermentation was "speeded up" until finally the reversion of reaction to give an alkaline test to methyl red took place within the customary time of four days. The production of acetyl methyl carbinol, as shown by a positive Voges-Proskauer test, was never apparent until this reversion had taken place. It will be seen from tables 2 and 3, C1 to C8, that the eight cultures which exhibited this peculiarity evidently belonged to the aerogenes-cloacae section of the group as judged by carbohydrate fermentations, Endo colonies, and by the utilization of both citrate and uric acid.

Irregular and atypical cultures in regard to the deportment toward the methyl red and Voges-Proskauer tests have been encountered by practically all who have worked with the colon group of organisms. As bearing especially upon the present investigation it might be mentioned that Chen and Rettger (1920) reported that 18 cultures resembling the aerogenes type gave at first methyl red positive, Voges-Proskauer positive tests. Upon repeated plating the number of non-correlating organisms was reduced to 4, although contaminations could not be demonstrated. They further state (p. 287) "The methyl red positive strains whose hydrogen-ion concentration was on the border line of the methyl red range (5.7 to 5.9) were made to return to their typical methyl red negative reactions by repeated plating, their pH values being raised to 6.2 to 6.5." Stovall and Nichols (1918) reported variation in the methyl red and Voges-Proskauer tests when they were applied twice at six-month intervals to 200 colon group organisms obtained from water supplies in the state of Wisconsin.

As previously pointed out, the methyl red positive soil cultures form an interesting group. (See tables 2 and 3, D1 to

D23, E1, E2). The carbohydrate fermentations of several of these cultures approached those assigned to the aerogenes-cloacae section (Winslow, Kligler, and Rothberg, 1919). On the other hand, the types of colonies formed on Endo medium resembled in many instances those shown by the typical fecal *Bact. coli*. It is a curious anomaly that most of the cultures which fermented salicin, sucrose, and raffinose, and thus approached the aerogenes type, should at the same time give a positive indol test (table 3, D2, to D6, D12 and D22). Again, the cultures which did not attack this series of carbohydrates and thus resembled the coli type were usually indol negative. Several of these cultures liquefied gelatin while others exhibited the delayed fermentation of lactose. Of the 25 methyl red positive soil cultures only 2 failed to develop in the citrate medium (tables 2 and 3, E1 and E2). These two resembled the typical fecal *Bact. coli* type in every respect. One of these cultures was obtained from a Virginia soil and one from a Maryland specimen taken about twelve miles from Washington, D. C. It is not known whether they represent true soil forms or whether in spite of precautions taken in collecting the samples, they indicate chance human or, more probably, animal pollution. The various cultural and differential tests point to the latter conclusion.

To correlate the source of the cultures with the various methods used for differentiation of the colon-aerogenes group, reference should be made again to tables 1 and 2. The fecal cultures of the *Bact. coli* type form an homogenous group with very little variation in any of these tests, all of which correlate almost perfectly. The fecal aerogenes cultures all utilized citrate and uric acid readily. However, upon examining the results obtained with the soil cultures one is immediately impressed, first, by the variability of the methyl red and Voges-Proskauer tests and, secondly, by the fact that 25 of a total of 72 cultures were found to be methyl red positive and Voges-Proskauer negative. The degree of correlation of the various differential tests is given at the foot of table 2. Here we find that 70 of 72 soil cultures, or 97.2 per cent, developed in the citrate medium. Only slightly over 50 per cent were consist-

ently Voges-Proskauer positive and methyl red negative, while 25, or 34.7 per cent, of the soil cultures were consistently methyl red positive and Voges-Proskauer negative. In the uric acid medium 43, or 59.7 per cent of the soil cultures were capable of development. In the present investigation then, the ability to utilize the citrate radical, as shown by growth in a synthetic citrate medium, was found to correlate very closely with the source of the cultures. The methyl red and Voges-Proskauer tests were of less value in placing the source of the cultures, since a fairly large proportion of the soil strains were methyl red positive and Voges-Proskauer negative. In addition, some variability and irregularity was encountered in the use of these tests. Uric acid appears to be less useful than citrate for only about 60 per cent of the soil cultures developed in the uric acid medium. It should be noted that most of the methyl red positive soil cultures which utilized citrate (table 2, D) refused to grow in the uric acid medium. Also a few of the typical aerogenes-cloacae soil types failed to show development in this medium.

It has been commonly assumed that all methyl red positive, Voges-Proskauer negative cultures encountered in soil, water, etc., are of fecal origin since about 95 per cent of fecal cultures give this reaction. However, in the present investigation 34.7 per cent of the colon group cultures from soil were methyl red positive and Voges-Proskauer negative and all but two of these cultures were distinct from the typical fecal *Bact. coli* as shown by their development in the citrate medium. There is evidence, therefore, that not all of the methyl red positive colon group cultures encountered in nature are of fecal origin. There is to be found in soil, in addition to the well-recognized aerogenes-cloacae type, another section of the colon group which may be characterized as being methyl red positive and Voges-Proskauer negative and possessing the ability to utilize citrate as a source of carbon. Hitherto this section has not been separated from the fecal methyl red positive type because the citrate differentiation has not been available. A suggestion that some of the methyl red positive colon group organisms encountered in soil

and water may differ from the fecal methyl red positive type is found in several recent publications. Thus, Chen and Rettger (1920) isolated from soil 20 methyl red positive colon cultures, 10 of which developed in the uric acid medium, whereas 173 colon strains isolated from feces gave negative results in this medium. Perry and Monfort (1921), in a study of atypical colon-aerogenes forms obtained from natural waters, found a number of methyl red positive organisms which also developed in the uric acid medium.

Whether the results presented in this paper will prove to have a practical application in the estimation of the sanitary quality of water remains to be seen. The methyl red and Voges-Proskauer tests, which at one time were regarded by many as solving all the difficulties encountered in interpreting the presence of the colon group in water, seem to have lost ground in recent years. Some investigators believe that in routine analysis this differentiation has little or no practical significance, while many water laboratories at the present time do not use it at all. On the other hand, others adhere to it and claim that it is useful in certain cases. Levine (1921) states that in routine water analysis this differentiation is obviously desirable as it may assist in the detection of the probable source and nature of the contamination. In the present investigation, the test of citrate utilization to distinguish types distinct from the fecal *Bact. coli* was found to constitute an additional differential characteristic which may possess some value in clearing up the present uncertainty. It has correlated much more closely with the source of the cultures than have any of the other methods of differentiation and it seems therefore that further study of the usefulness of the citrate differentiation would be warranted, especially as applied to cultures obtained from waters of different sanitary quality.

SUMMARY

In a series of colon-aerogenes cultures, the source of the organisms was compared with the various differential tests used to separate the sections of this group. Of the 190 cultures em-

ployed, 118 were isolated from fecal specimens of both man and animals and 72 were obtained from various soil samples which were collected from locations where the chance of pollution seemed remote. The differential tests employed were, first, determination of the ability to utilize citrate as a source of carbon, secondly, the ability to utilize uric acid as a source of nitrogen and, lastly, the usual methyl red and Voges-Proskauer tests.

The results confirmed previous work in that the typical *Bact. coli* of fecal origin is unable to utilize sodium or potassium citrate when supplied as the sole source of carbon, whereas the aerogenes-cloacae types, which represented the largest section of the soil cultures, all utilized citrate readily.

Some exceptions were noted to the usual correlation between the manner of glucose metabolism, as shown by the methyl red and Voges-Proskauer tests, and the ability to utilize citrate. These discrepancies occurred chiefly among the soil cultures. Of especial interest were 23 soil strains which were consistently methyl red positive and at the same time developed in the citrate medium. These organisms seem to constitute another section of the colon-aerogenes group which may be differentiated from the fecal methyl red positive type by their utilization of citrate. Their presence in soil and apparent absence from fecal specimens constitutes evidence that not all of the methyl red positive colon group organisms encountered in nature are of fecal origin.

The citrate medium appeared to advantage in separating the various types of the colon group from the typical fecal *Bact. coli* and the test of citrate utilization correlated more closely with source than did any of the other differential tests. Thus, of the 72 soil cultures, 70, or 97.2 per cent, utilized citrate, slightly over 50 per cent were consistently alkaline to methyl red and Voges-Proskauer positive, while about 60 per cent developed in the uric acid medium.

Irregularity and variability of the methyl red and Voges-Proskauer tests were encountered in a number of cultures. Differentiation by means of the citrate medium was found to be especially useful in assigning these cultures to their proper groups as well as indicating their source.

The results obtained in the present investigation suggest that the test of citrate utilization deserves further study with respect to the distribution of the colon-aerogenes group in nature, with especial reference to possible usefulness in the estimation of the sanitary quality of water.

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THE CHARACTER OF ACIDS PRODUCED BY HEMOLYTIC AND NON-HEMOLYTIC STREPTOCOCCI FROM PATHOGENIC SOURCES AND FROM MILK

I. INTRODUCTION

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Studies of fermentation reactions by the streptococcus group have been approached from several angles. Early workers headed by Gordon (1904) sought a differentiation based on the ability or inability of the organisms to ferment certain substances. Later, the amount of acid produced, as determined by titration, was considered of importance. More recently the work of Clark and Lubs on the differentiation of members of the colon group by the determination of the limiting hydrogen-ion concentration has led to the application of this phase of fermentation study to the streptococci. So far, little attention has been paid to the products of fermentation or to the kinds of acids produced. Evans (1914 and 1918) has studied the substances formed by five strains of streptococci concerned with cheese ripening, and Foster (1921) has thoroughly investigated the fermentative and putrefactive products of one strain of *Streptococcus hemolyticus* from a fatal case of bronchopneumonia.

The purpose of the present investigation was to determine the character and ratio of the acids produced by hemolytic streptococci with the hope of discerning distinctive differential features in their acid products. The study included first, an identification of the organisms, second, a comparison of titratable acidity with limiting hydrogen-ion concentration, third, a determination of the proportion of volatile and non-volatile acids with an investigation of the amounts of each produced daily,

and fourth, a qualitative, quantitative, and graphical study of the kind of acids formed.

II. METHODS

a. Sources of cultures

Cultures from pathogenic conditions were obtained from the bacteriological laboratory of the Johns Hopkins Hospital during the months of June and July, 1922, from the Hygienic Laboratory in Washington, from the laboratory of H. K. Mulford Company, and from stock cultures at the bacteriological laboratory of the Johns Hopkins School of Hygiene. The streptococci from milk were obtained by plating samples of unpasteurized milk secured from one of the dairies in Baltimore during the month of July, 1922.

b. Media employed

Pure strains of streptococci were transferred to Huntoon's infusion broth, incubated eighteen hours, then plated on blood agar (0.5 cc. citrated rabbit's blood, 10 cc. infusion agar). Hemolysis was noted here, but again confirmed in infusion broth tubes which were incubated for eighteen hours, and then after the addition of 0.2 cc. of a 5 per cent solution of rabbit or sheep cells incubated for one hour longer.

Colonies were fished from plates to blood agar slants (0.2 cc. rabbit's blood, 5 cc. infusion agar), and incubated for twenty-four hours. Tubes of Huntoon's plain broth were inoculated from the blood agar slants, and incubated eighteen to twenty-four hours. The initial pH of all the broth used was 7.2. Later in the work, infusion broth was substituted for Huntoon's unfiltered broth, but no marked differences were noted in the final results obtained. One per cent lactose, sucrose, and glucose were used throughout all the experiments.

Five-tenths cubic centimeter of a culture in Huntoon's plain broth was used to inoculate flasks containing 100 cc. Huntoon's broth plus 1 per cent of sugar. The sterile sugars were added after the flasks containing the broth had been autoclaved, in

order that no caramelization of the sugar should take place. The broth used with each sugar was made from the same meat and titrated in the same container, so that its salt content and hydrogen-ion concentration were uniform.

The flasks were provided with sterile rubber stoppers to prevent evaporation, and were uniform in size and shape, so that the surface exposed was the same in all. They were incubated until fermentation was complete but each day the amount of acid formed was determined and the reaction readjusted. The flasks were always returned to their same positions in the incubator, incubated at 37°C., and examined each day at the same hour, so that as nearly as possible all conditions would be alike.

c. Titration of broth cultures in flasks

Five-tenths cubic centimeter of culture was removed from each flask with a sterile pipette, and added to small tubes containing 2 cc. sterile distilled water, and 0.2 cc. phenolsulphonephthalein indicator. To 0.5 cc. of the culture, N/20 KOH was added drop by drop until the pH value, as indicated by comparison with standard buffer solutions, was the same as that of the original broth. The amount of N/1 KOH to be added to bring the flask as a whole back to pH 7.2 was then calculated, always taking into consideration the 0.5 cc. of the culture withdrawn for the tests each day. This procedure was followed until no more acid was produced by the streptococci. The streptococci usually were still living at this point, as was shown by plating a sample of the broth, but were unable to produce further acid since the sugar was completely used up.

Direct pH determinations were also made with attempted computation of acid formation based on buffer curves especially determined for the original broth. This method was found to work very well for the first few days of titration, but after that could not be relied upon, due to a physical change in the buffer of the broth, to a mixture of acids of varying ionizing properties being formed by the streptococci, and to the potassium salts formed by the addition of the alkali.

Titrations in value of N/1 KOH for each day were added, to give the *total acidity*, including volatile and non-volatile acids. All possible precautions were taken to avoid contamination when removing the sample from the flask and in adding the alkali. Plates were made from the culture to insure purity, once in the middle of the experiment, and again at the end when no further acid was produced.

d. Steam distillation

The separation of volatile and non-volatile acids was effected by the steam distillation method of Ducleux (1900) as modified by Dyer (1916). The entire culture was transferred to a large distilling flask, and 200 cc. of distilled water added. Ten drops of concentrated sulphuric acid was added to liberate the volatile acids from their potassium salts, and a small piece of paraffin added to lessen the foaming. The apparatus was set up as described by Dyer (1916) and the distillation carried on until 500 cc. of distillate had passed over.

The entire distillate was titrated with N/20 KOH using phenol-sulphonephthalein as indicator and the result then converted into terms of N/1 KOH.[1] The result obtained from the titration of the distillate, indicating the amount of volatile acids present, was subtracted from the value of the total acidity, and divided into the remainder to give the ratio of volatile to non-volatile acids.

III. DISCUSSION OF RESULTS

The titrations of the first and second day showed nearly twice as much acid production by the non-hemolytic streptococci as by the hemolytic, regardless of which sugar broth was fermented. The final total acidity varied but little in each sugar, although the highest acidity in all but a very few cases was produced in glucose broth. The growth of the hemolytic streptococci was maintained longer than that of the non-hemolytic strains; they fermented a smaller amount of sugar each day, but

[1] Test for possible sulphates which might have been carried over into the distillate was made with barium hydroxide, but none were found.

in the end the total acidity was about equal. Table 1 shows characteristic results for representative hemolytic and non-hemolytic organisms.

Records of acid production by hemolytic and non-hemolytic streptococci in glucose, lactose, and sucrose broths, with the ratios of volatile to non-volatile acid content found in each, were derived from the averages of the volatile and non-volatile

TABLE 1

Comparison of pH values with daily acid production by hemolytic and non-hemolytic streptococci

NUMBER OF DAYS	HEMOLYTIC 133a STREP. HEMOLYTICUS II (HOLMAN)		NON-HEMOLYTIC J-5 STREP. FECALIS (HOLMAN)	
	pH	Normal acid	pH	Normal acid
		cc.		cc.
1	6.2	1.96	5.6	3.48
2	6.4	1.46	5.8	2.97
3	6.4	1.45	6.2	1.97
4	6.4	1.45	6.4	1.47
5	6.4	1.44	6.6	0.97
6	6.6	0.96	6.6	0.97
7	6.6	0.95	7.0	0.48
8	6.6	0.95	7.2	
9	7.0	0.47	7.2	
10	7.0	0.47		
11	7.0	0.46		
12	7.1	0.23		
13	7.2			
14	7.2			
15	7.1	0.22		
16	7.2			
17	7.2			
		12.45		12.31

acid production found in four duplicate experi nents performed at the same time, under like conditions with flasks inoculated with equal amounts of the same seed cultures and grown in broth from one infusion.

The experiments indicated little constancy even for the same strain of organisms; nor could any significance be attached to the sources from which the hemolytic streptococci had come.

The respective ratios for volatile and non-volatile acid production by hemolytic and non-hemolytic streptococci were grouped in their ascending values, it was found, however, that the non-hemolytic streptococci had a very wide range, while the hemolytic streptococci were nearly all confined to the lower ratios, below one part of volatile to seven parts of non-volatile acid.

The observed ratios may be arranged in percentage groups as follows:

	PER CENT OF HEMOLYTIC STREPTOCOCCI BELOW RATIO 1:7	PER CENT OF NON-HEMOLYTIC STREPTOCOCCI BELOW RATIO 1:7
Glucose	71	46
Lactose	91	52
Sucrose	79	44

The averages of all duplicate determinations of total acidities and volatile acid content were taken, and the ratios between the averages found. This smoothed out many of the erratic ratios, for which no explanation could be given, and still showed clearly the great range which the non-hemolytic strain covered, and the narrow limits of the hemolytic streptococci.

	PER CENT OF HEMOLYTIC STREPTOCOCCI BELOW RATIO 1:7	PER CENT OF NON-HEMOLYTIC STREPTOCOCCI BELOW RATIO 1:7
Glucose	92	41
Lactose	100	54
Sucrose	100	42

The ratios obtained from cultures of hemolytic and non-hemolytic streptococci were then analyzed by comparing the mean ratios and their probable errors. The number of ratios of volatile to non-volatile acids recorded for each sugar represents the total number of averages from four duplicate determinations. It had been hoped that an acid ratio, comparable to the gas ratio might be evolved from these computations. But the great overlapping of each group made it impossible, although the

results clearly show a distinct difference between the ratio of volatile to non-volatile acid production by hemolytic and non-hemolytic streptoccci.

Studies on daily changes in the ratio of volatile to non-volatile acid were made as follows: Flasks containing 1 liter of 1 per cent sugar infusion broth were inoculated with 5 cc. of a twenty-four hour infusion broth culture. One hundred cubic centimeter were removed each day, titrated, and distilled, the remainder in the flask was adjusted to the original pH of the

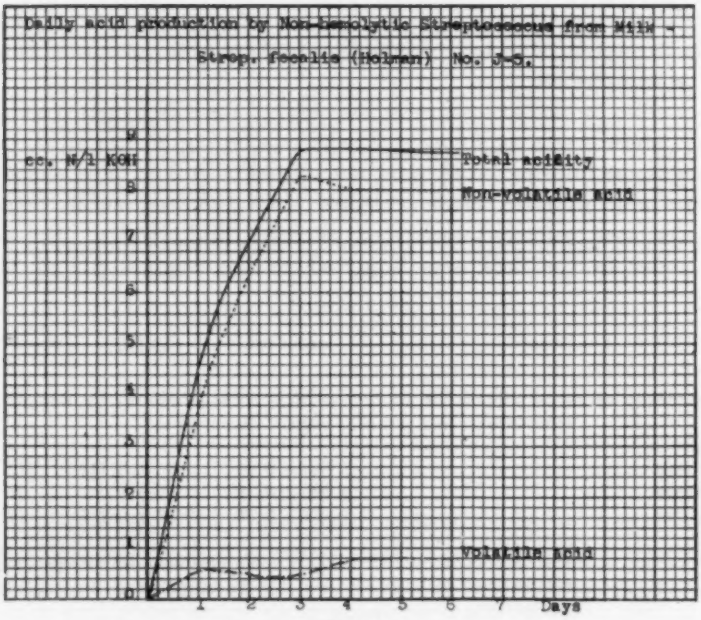
SUGAR	MEAN RATIO	STANDARD DEVIATION	TOTAL NUMBER OF RATIOS
Frequency of ratios of volatile to non-volatile acids produced by hemolytic streptococci in 1 per cent sugar infusion broth			
Sucrose	5.743 ± 0.211	1.9 ± 0.149	37
Glucose	5.398 ± 0.197	2.43 ± 0.139	69
Lactose	4.361 ± 0.29	2.58 ± 0.205	36
Frequency of ratios of volatile to non-volatile acids produced by *non-hemolytic* streptococci in 1 per cent sugar infusion broth			
Sucrose	7.796 ± 0.203	2.9 ± 0.143	54
Glucose	7.694 ± 0.233	3.54 ± 0.166	104
Lactose	7.833 ± 0.547	4.87 ± 0.387	36

broth. This procedure was carried on until no further acid production was found to occur. This method proved satisfactory. (See graphs I and II.)

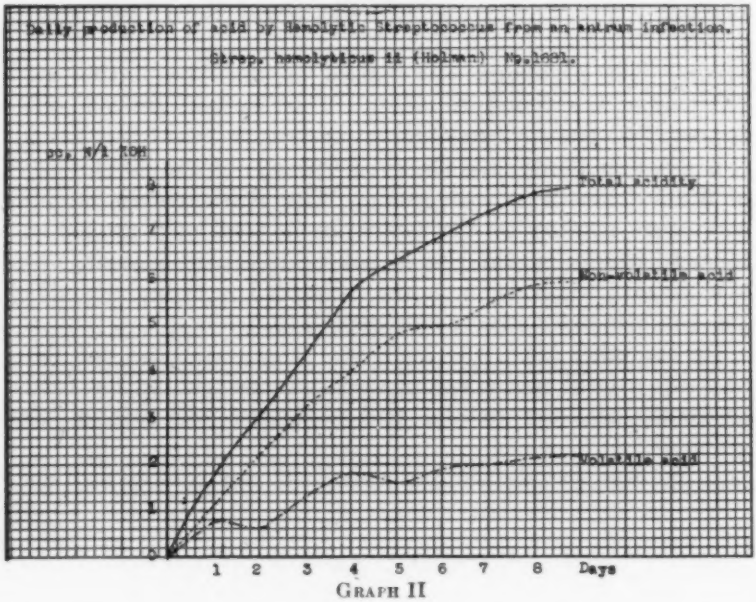
A great similarity was observed throughout the development of the individuals in each group. The non-hemolytic streptococci were marked by the rapid rise in total acid production, followed closely by the rise in non-volatile acid content. The volatile acid formation was low. Graph I is typical for all of the non-hemolytic streptococci examined.

On the other hand, the hemolytic streptococci did not show as steep a total acid curve, the non-volatile acid did not follow as closely, and the volatile acid production was greater. (See graph II.)

In no case was the ratio of volatile to non-volatile acid con-


Daily acid production by Non-Hemolytic Streptococcus from Milk -
Strep. faecalis (Holman) No. J-S.
cc. N/2 KOH
Total acidity
Non-volatile acid
Volatile acid
1 2 3 4 5 6 7 Days

GRAPH I


Daily production of acid by Hemolytic Streptococcus from an enteric infection.
Strep. hemolyticus #1 (Holman) No.1008.
cc. N/2 KOH
Total acidity
Non-volatile acid
Volatile acid
1 2 3 4 5 6 7 8 Days

GRAPH II

stant throughout the experiments, as a glance at the graphs and the table below will show. In this respect we failed to confirm the results of Fred, Peterson and Davenport (1919).

GRAPH NUMBER	CULTURE NUMBER	RATIOS OF DAILY PRODUCTION OF VOLATILE TO NON-VOLATILE ACIDS							
		1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
I	J-5	1 to 5.9	12.1	17.2	12.3	12.3			
II	1681	1.4	3.3	2.5	2.0	3.1	2.6	2.7	2.7

IV. CHARACTER OF ACIDS PRODUCED

a. Volatile acids

In 1921, Foster (1921) working with a culture of *Strep. hemolyticus* showed that lactic acid appeared to be the principal acid formed in its fermentation of glucose broth. A smaller proportion of volatile acid was formed, made up chiefly of acetic, with perhaps a trace of formic acid.

Evans (1914 and 1918) made a study of the volatile acids produced by two cheese streptococci and *Strep. lacticus*. Some of her data indicated traces of acids of lower molecular weight than acetic —probably formic —and also traces of acids higher than acetic, possibly butyric, which might arise from a slight hydrolysis of the fat in the milk. It seemed justifiable to conclude that acetic acid was the only volatile acid produced by either *Strep. lacticus* or the cheese streptococci in their normal process of metabolism in milk culture.

Like Foster (1921) I found the color tests suggested by Dyer to be neither successful nor reliable. Various other qualitative tests were therefore applied to the distillate in an attempt to determine the volatile acids present.

1. Microscopic examination of crystals. Calcium salts were formed by evaporating the neutralized distillate to a volume of 25 cc., acidifying with a few drops of sulphuric acid, warming to liberate any carbon-dioxide present, neutralizing with ammonia, and adding a solution of calcium chloride. This was

heated to boiling, and a drop put on a slide under the microscope to compare the form of the crystals with those of the known calcium salts of the lower volatile fatty acids. This method was fairly successful but the formation of the crystals had to be carefully watched as they developed, for many forms were observed in their progressive stages. Calcium acetate was found to predominate, while occassionally calcium butyrate crystals appeared.

2. Preparation of volatile ester. The volatile esters of the acids were made by following the directions of Mullikan (1918), which consisted in adding 0.1 cc. concentrated sulphuric acid to the evaporated neutralized distillate, slowly warming and noting the odor of the vapors of the liberated organic acid. The tube was cooled, and 0.2 cc. strong ethyl alcohol added. This was warmed until the vapors again came off freely. The tube was then poured into a watch glass containing 5 cc. cold water where the odors were more easily observed than in a test tube. The test is rather delicate, and if made comparably may suggest which of the four acids are present. The volatile esters of the acids strongly indicated the presence of both acetic and propionic acids with a slight suggestion of ethyl butyrate in some cultures.

Since the preceding determinations did not include an indicatory test for formic acid, a special procedure was carried out for that acid.

3. Test for formic acid. A test for unsaturated acids was applied to a 10 cc. portion of the distillate which had not previously been titrated. A few crystals of sodium carbonate were added to the solution until it became alkaline. A 1 per cent solution of potassium permanganate was added drop by drop. If the purple color of the permanganate was instantly destroyed, formic acid was present. A reduction of potassium permanganate was obtained in every case where the distillate of a non-hemolytic streptococcus was tested. Negative results were always obtained from the distillates of hemolytic streptococci and *Strep. viridans.*

Although the last two tests had proved successful, it seemed best to make a graphical representation of the volatile acids as fractionally distilled, and compare the curves of the unknown mixtures with the graphs of known acids.

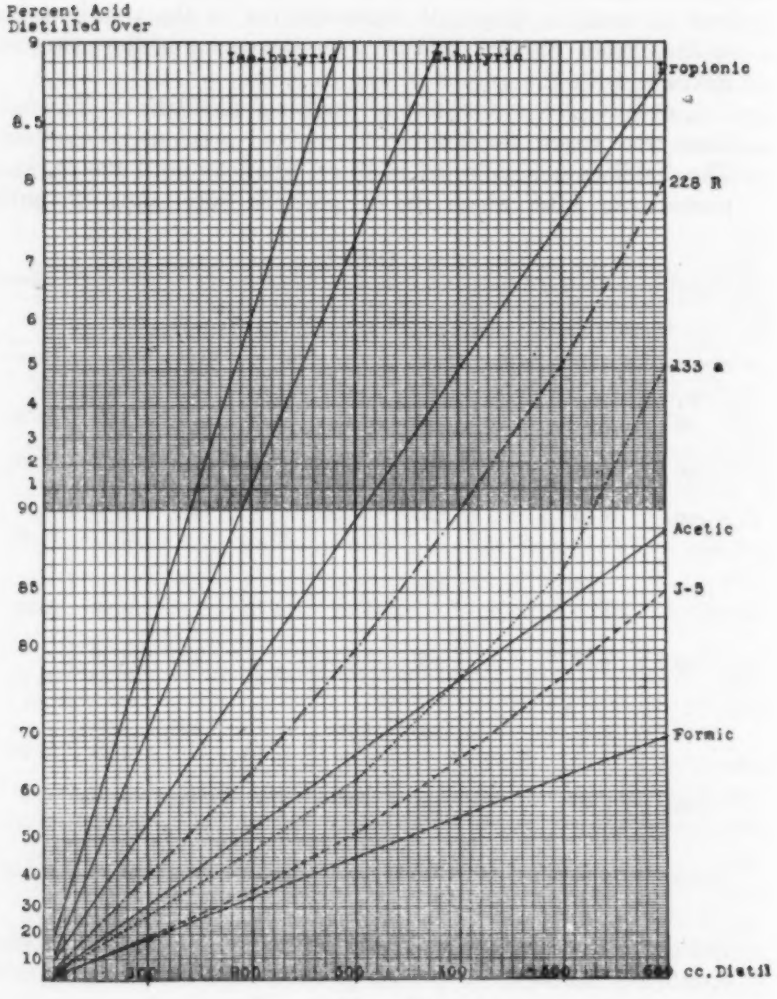
4. Graphical representation of fractional distillation. The cultures were steam distilled by the method previously described. The distillate was collected in 10 cc. fractions until 100 cc. had passed over, after which 100 cc. portions were taken off, until

TABLE 2

Distilling constants of volatile acids from sugar infusion broth cultures of streptococci

J-5 NON-HEMOLYTIC			133a HEMOLYTIC			229 S. STREP. VIRIDANS		
Fraction	N/20 acid	Per cent	Fraction	N/20 acid	Per cent	Fraction	N/20 acid	Per cent
cc.	cc.		cc.	cc.		cc.	cc.	
10	0.9	1.86	10	1.45	1.96	10	3.05	4.74
100	8.60	17.87	100	19.52	26.50	100	25.3	39.98
200	8.50		200	14.37		200	15.51	
	17.10	35.54		33.89	46.00		40.81	63.50
300	7.70		300	11.79		300	10.61	
	24.80	51.54		46.68	62.00		51.42	80.00
400	7.00		400	11.42		400	6.43	
	31.80	66.09		57.10	77.49		57.85	89.95
500	3.55		500	6.30		500	3.21	
	37.35	77.63		63.40	86.04		61.06	95.00
600	3.55		600	6.60		600	1.94	
	40.90	85.00		70.00	95.00		63.00	98.00
3,500	48.11	100.00	2,500	73.00	100.00	1000	64.28	100.00

600 cc. of the distillate had been secured. The distillation was continued, taking only 500 cc. portions, until all volatile acid was entirely removed from the culture. These fractions were then titrated with N/20 KOH, using phenolphthalein as indicator, and the percentage of acid calculated. By plotting a curve from the


Percent Acid
Distilled Over
9
8.5
8
7
6
5
4
3
2
1
90
85
80
70
60
50
40
30
20
10
Iso-butyric
N-butyric
Propionic
228 R
233 a
Acetic
J-5
Formic
cc. Distil

GRAPH III

distilling constants of the distillate of an unknown acid mixture, and comparing it with the curves established for known acids, the individual acids may be approximately determined. The logarithmic chart, graph III, shows in solid lines the straight paths of known acids calculated by Dyer (1916) and plotted from his values of constants. The curves plotted from the distilling constants (shown in table 2) proved to be so closely analogous to the results of the other representatives of the larger groups of streptococci, that only one graph (graph III) will be shown. The curved paths are characteristic for three groups of streptococci, namely, the hemolytic, non-hemolytic and viridans types. The hemolytic streptococci isolated from milk gave the characteristic curve of the pathogenic organisms.

It was realized that this method for analysis of acid mixtures has its limitations. It can readily be observed that a single steam distillation unsupported by sense of smell, or other tests, would not be sufficient to determine the number of acids present in an unknown mixture, unless the number were two, and these were adjacent acids, or acids such as formic and butyric for which distinctive qualitative tests are available.

The broken line curves in the logarithmic chart, however, in general substantiate the previous qualitative observations. The distillates from cultures of non-hemolytic streptococci—represented by curve J-5—always gave a strong reaction for formic acid, and the addition of alcohol and sulphuric acid gave a moderately strong odor of ethylacetate. The slow rate of distillation also substantiated the presence of formic acid. (See table 2.)

The distillates from cultures of hemolytic streptococci, represented by curve 133a, indicated the presence of formic acid although all qualitative tests applied to distillates of hemolytic streptococci were negative for that acid. As it is difficult to distinguish between the esters of propionic and acetic acid, it can only be said that either one or the other, or both were present, with a slight additional suggestion of butyric.

The cultures of *Strep. viridans* distilled more rapidly than the cultures of the hemolytic or non-hemolytic streptococci giving

a higher percentage of acid for each fraction. Qualitatively, the distillate was the same as 133a, but graphically it showed closer proximity to propionic acid.

b. Non-volatile acids

1. Formation of barium salts. Gravimetric determination of the kind and proportion of non-volatile acid was made for two hemolytic and two non-hemolytic cultures. The results show lactic acid to be the chief non-volatile acid present. The differences between the calculated amount of normal lactic acid and the amount of non-volatile acid actually found in the cul-

TABLE 3

Lactic acid as determined by barium salt method

	WEIGHT OF Ba SALTS	WEIGHT OF $BaSO_4$	CALCULATED AMOUNT LACTIC ACID	ACTUAL AMOUNT OF NON-VOLATILE ACID TITRATED IN CULTURE
	grams	*grams*	*cc.*	*cc.*
Hemolytic:				
237L-3	0.98	0.71	6.18	6.64
133a-3	1.46	1.06	9.31	9.90
Non-hemolytic:				
J-7-4	1.52	1.12	9.77	10.40
D 1	1.50	1.09	9.50	9.71

ture by the subtraction of the volatile acid from the total acidity, may be due to the presence of another non-volatile acid in small amounts, or to experimental error accentuated by the repeated filterings necessary to clear the solution of the elements of broth which constantly clouded it.

2. Formation of zinc salts with determination of rotary type. The zinc salts of lactic acid have been used by a number of investigators to determine both the quantity and the form of acid produced. Fred and Peterson (1919) state that in the spontaneous fermentation of sugar solution the usual form of lactic acid produced is the racemic type. One means of determining the form of acid is by finding the amount of water of crystallization contained in the zinc salt. The racemic zinc lactate crystallizes with three molecules of water, constituting

18.7 per cent of the weight of the salt, and the active zinc lactate with two molecules, forming 12.9 per cent of the weight. By heating several hours in an electric oven at 105° to 108° this water of crystallization was driven off. By difference in weight before and after heating the percentage loss of water was calculated. Two determinations were made to find the percentage of water of crystallization in the zinc salt. 17.2 per cent and 17.7 per cent of the weight of the samples were water, showing that it was the racemic zinc lactate which crystallizes with three molecules of water and theoretically constitutes 18.7 per cent of the weight of the salt.

V. CONCLUSIONS

1. During the first twenty-four hours of growth in a sucrose, or lactose infusion broth, the acid production by non-hemolytic streptococci was found to be nearly twice as great as the acid production by hemolytic streptococci.

2. Titratable acidity and hydrogen-ion content are only comparable in cultures grown in the same lot of broth and during the early stages of fermentation.

3. Total acidity at the end of the acid production period in 100 cc. 1 per cent sugar infusion broth, was about equal in the two groups of streptococci.

4. All hemolytic streptococci showed a gradual rise in daily acid production with a low ratio of volatile to non-volatile acid content.

5. All non-hemolytic streptococci were alike in showing a rapid rise in total acidity during the first days of fermentation, with a low production of volatile acid, giving a high ratio of volatile to non-volatile acid content.

6. The ratio of volatile to non-volatile acid was determined for 30 non-hemolytic and 32 hemolytic streptococci. The mean ratio for the hemolytic streptococci was 1 to 5.167 with a comparatively small deviation. The mean ratio for the non-hemolytic streptococci was 1 to 7.773 with a wide deviation.

7. The non-volatile acid produced was almost entirely lactic acid of the racemic type.

8. Non-hemolytic streptococci produced formic, acetic, and a possible trace of butyric acid.

9. Hemolytic streptococci and *Strep. viridans* produced acetic and propionic acids. Butyric acid was probably present in small quantities. All tests for formic acid were negative.

10. The acids produced by hemolytic streptococci isolated from milk were like those formed by the hemolytic streptococci which had come from pathogenic sources.

11. Streptococci isolated from similar pathogenic lesions or bearing like specific names, showed no constancy of ratio between the volatile and non-volatile acids which they produced.

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